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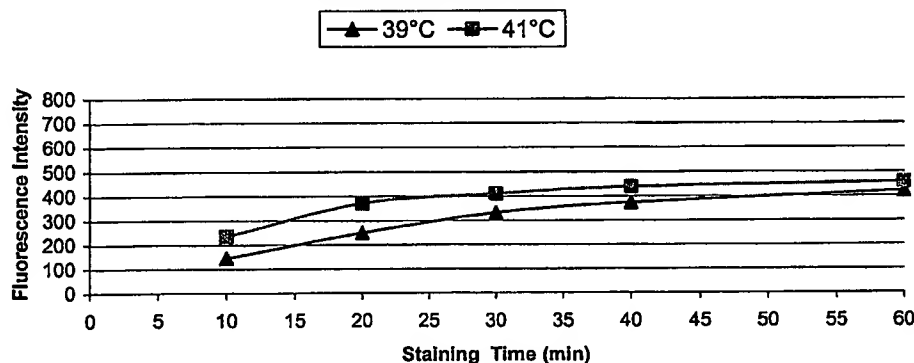
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(54) Title: PROCESS FOR THE STAINING OF SPERM

Comparison of Fluorescence Intensity of sperm stained with 20uM  
Hoechst 33342 at 39°C & 41°C



(57) Abstract: Sperm cells are stained according to processes that involve the combining of sperm cells with a fluorescent DNA selective dye at an elevated temperature in excess of about 40°C. The methods allow for a decreased staining time. The cells may thereafter be efficiently sorted according to common separation methods, including flow cytometry.

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*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

**PROCESS FOR THE STAINING OF SPERM****Background of the Invention**

[0001] The present invention generally relates to a  
5 staining process to enable sperm separation for the purpose  
of producing gender enriched sperm.

[0002] The fertilization of animals by artificial  
insemination (AI) and embryo transplant following *in vitro*  
fertilization is an established practice. In the livestock  
10 production industry, the ability to influence the  
reproductive outcome toward offspring having one or more  
desired characteristics has obvious advantages. By way of  
example, there would be an economic benefit in the dairy  
industry to preselect offspring in favor of the female sex  
15 to ensure the production of dairy cows. The separation of  
sperm into enriched populations of X and Y chromosome-  
bearing cells, known as gender enriched semen or gender  
enriched sperm, is one method of achieving preselected  
offspring.

20 [0003] Johnson et al. (U.S. Patent No. 5,135,759)  
describe the separation of intact X and Y chromosome-  
bearing sperm populations according to DNA content using a  
flow cytometer/cell sorter into X and Y chromosome-bearing  
sperm enriched populations. As described, the sperm is  
25 combined with a DNA selective dye at a temperature of 30 to  
39 °C for a period of 1 hour (39 °C) to 1.5 hours (30 °C).  
A flow cytometer is then used to measure the amount of  
fluorescent light given off when the sperm passes through a  
laser beam. Because the X chromosome-bearing sperm  
30 contains more DNA than the Y chromosome-bearing sperm,  
approximately 3 to 5% depending upon the species, the X  
chromosome-bearing sperm yields a greater intensity of  
fluorescent light than the Y chromosome-bearing sperm.  
Droplets containing single sperm of a predetermined  
35 fluorescent intensity are given a charge and

electrostatically deflected into collection vessels. The collected, gender enriched sperm population, is then used for microinjection or artificial insemination.

[0004] Seidel et al. (WO 02/43574) also describe  
5 separation of sperm into gender enriched populations of X and Y chromosome-bearing cells using flow cytometry. Seidel et al. describe staining the cells at a temperature between 30°C and 40°C.

[0005] Didion et al. (WO 02/41906) describe staining  
10 sperm cells at temperatures of about 17°C to 30°C. According to Didion et al., these staining temperatures avoided certain effects which may result from staining at greater temperatures, such as reduced sperm viability and efficiency. Furthermore, it was believed that the lower  
15 temperature staining provided advantageous effects on sperm orientation during sorting.

#### Summary of the Invention

[0006] Among the various aspects of the present  
20 invention is the provision of a relatively rapid and efficient method of staining sperm cells and the provision of such a process in which decreased periods are required for dye uptake, thereby decreasing the time that elapses  
25 between the time of semen collection and production of gender enriched populations of X and Y chromosome-bearing sperm cells.

[0007] Briefly, therefore, the present invention is directed to a process for staining sperm cells. The  
30 process comprises forming a staining mixture containing intact viable sperm cells and a DNA selective fluorescent dye, and subjecting the staining mixture to a temperature of at least about 40°C.

[0008] Other aspects of the invention will be in part  
35 apparent, and in part pointed out hereinafter.

**Brief Description of the Drawings**

[0009] FIGURES 1A - 1D graphically depict the results of the study carried out in Example 1 wherein fluorescence intensity of sperm is measured for sperm stained at varying concentrations of Hoechst 33342 dye at 39°C and 41°C.

[0010] FIGURES 2A - 2D graphically depict the results of the study carried out in Example 2 wherein fluorescence intensity of sperm is measured for sperm stained at varying concentrations of Hoechst 33342 dye at 39°C and 41°C.

[0011] FIGURES 3A - 3D graphically depict the results of the study carried out in Example 3 wherein fluorescence intensity of sperm is measured for sperm stained at varying concentrations of Hoechst 33342 dye at 39°C and 43°C.

[0012] FIGURES 4A and 4B graphically depict the results of the study carried out in Example 4 wherein fluorescence intensity of sperm is measured for sperm stained at varying concentrations of Hoechst 33342 dye at 39°C and 43°C.

[0013] FIGURES 5A and 5B graphically depict the results of the study carried out in Example 5 wherein fluorescence intensity of sperm is measured for sperm stained at varying concentrations of Hoechst 33342 dye at 39°C and 45°C.

[0014] FIGURES 6A and 6B graphically depict the results of the study carried out in Example 6 wherein fluorescence intensity of sperm is measured for sperm stained at varying concentrations of Hoechst 33342 dye at 39°C and 47°C.

[0015] FIGURES 7A and 7B graphically depict the results of the study carried out in Example 7 wherein percent motility and percent progressive motility of sperm are measured for sperm unstained or stained with 60 $\mu$ M Hoechst 33342 dye at 39°C and 43°C.

[0016] FIGURES 8A and 8B graphically depict the results of the study carried out in Example 8 wherein percent motility and percent progressive motility of sperm are measured for sperm unstained or stained with 60 $\mu$ M

5 Hoechst 33342 dye at 39°C and 45°C.

[0017] FIGURES 9A - 9C graphically depict the results of the study carried out in Example 9 wherein percent motility and percent progressive motility of sperm are measured for sperm unstained or stained with 80 $\mu$ M Hoechst

10 33342 dye at 41°C, 43°C, and 45°C.

[0018] FIGURES 10A - 10C graphically depict the results of the study carried out in Example 10 wherein percent motility and percent progressive motility of sperm are measured for sperm unstained or stained with 100 $\mu$ M

15 Hoechst 33342 dye at 41°C, 43°C, and 45°C.

[0019] FIGURE 11 graphically depicts the results of the study carried out in Example 11 wherein percent motility and percent progressive motility of sperm are measured for sperm unstained or stained with 100 $\mu$ M Hoechst

20 33342 dye at 47°C.

[0020] FIGURE 12 graphically depicts the results of the study carried out in Example 12 wherein percent motility and percent progressive motility of sperm are measured for sperm unstained or stained with 100 $\mu$ M Hoechst

25 33342 dye at 49°C.

[0021] FIGURES 13A and 13B graphically depict the results of the study carried out in Example 13 wherein percent motility and percent progressive motility of sperm are measured for sperm unstained, stained with 125 $\mu$ M

30 Hoechst 33342 dye, or stained with 150 $\mu$ M Hoechst 33342 dye at 43°C.

[0022] FIGURES 14A and 14B graphically depict the results of the study carried out in Example 14 wherein

percent motility and percent progressive motility of sperm are measured for sperm unstained, stained with 200 $\mu$ M Hoechst 33342 dye, or stained with 250 $\mu$ M Hoechst 33342 dye at 43°C.

5        [0023] FIGURES 15A and 15B graphically depict the results of the study carried out in Example 15 wherein percent motility and percent progressive motility of sperm are measured for sperm unstained, stained with 125 $\mu$ M Hoechst 33342 dye, or stained with 150 $\mu$ M Hoechst 33342 dye  
10    at 45°C.

      [0024] FIGURES 16A - 16C graphically depict the results of the study carried out in Example 16 wherein percent motility and percent progressive motility of sperm are measured for sperm unstained, stained with 200 $\mu$ M  
15    Hoechst 33342, stained with 250 $\mu$ M Hoechst 33342, or stained with 350 $\mu$ M Hoechst 33342 at 45°C.

      [0025] FIGURES 17A - 17C graphically depict the results of the study carried out in Example 17 wherein percent motility and percent progressive motility of sperm  
20    are measured for sperm unstained, stained with 300 $\mu$ M Hoechst 33342, stained with 350 $\mu$ M Hoechst 33342, or stained with 400 $\mu$ M Hoechst 33342 at 43°C.

      [0026] FIGURES 18A - 18C graphically depict the results of the study carried out in Example 18 wherein  
25    percent motility and percent progressive motility of sperm are measured for sperm unstained, stained with 0.6 $\mu$ M SYBR 14, stained with 6.0 $\mu$ M SYBR 14, or stained with 60 $\mu$ M SYBR at 43°C.

      [0027] FIGURES 19A - 19B graphically depict the  
30    results of the study carried out in Example 19 wherein fluorescence intensity of sperm is measured for sperm stained with 100  $\mu$ M bisbenzimidazole-BODIPY conjugate (BBC) or 6 $\mu$ M SYBR 14 at 39°C and 45°C.

[0028] FIGURE 20 graphically depicts the results of the study carried out in Example 20 wherein percent progressive motility of sperm is measured for sperm stained with 400 $\mu$ M Hoechst 33342 dye at 41°C in either a TCA buffer or a TCA buffer containing 10mM pyruvate.

[0029] FIGURE 21 graphically depicts the results of the study carried out in Example 21 wherein percent progressive motility of sperm is measured for sperm stained with 400 $\mu$ M Hoechst 33342 dye at 41°C in either a TCA buffer or a TCA buffer containing 10 $\mu$ M vitamin K.

[0030] FIGURE 22 graphically depicts the results of the study carried out in Example 22 wherein percent progressive motility of sperm is measured for sperm stained with 400 $\mu$ M Hoechst 33342 dye at 41°C in either a TCA buffer or a TCA buffer containing 100 $\mu$ M vitamin K.

[0031] FIGURE 23 graphically depicts the results of the study carried out in Example 23 wherein percent progressive motility of sperm is measured for sperm stained with 400 $\mu$ M Hoechst 33342 dye at 41°C in either a TCA buffer or a TCA buffer containing 1mM lipoic acid.

[0032] FIGURE 24 graphically depicts the results of the study carried out in Example 24 wherein percent progressive motility of sperm cells is measured for sperm cells stained with 300 $\mu$ M Hoechst 33342 dye at 41°C in TCA containing 10mM pyruvate and then diluted 1 to 3 with either TCA containing 10mM pyruvate or a carbonate-based inhibitor at pH 6.2.

[0033] FIGURE 25 graphically depicts the results of the study carried out in Example 24 wherein percent progressive motility of sperm cells is measured for sperm cells stained with 300 $\mu$ M Hoechst 33342 dye at 41°C in (1) TCA containing 10mM pyruvate and diluted 1 to 3 with the same or (2) a carbonate-based buffer at pH 7.3 and diluted 1 to 3 with carbonate-based inhibitor at pH 6.2.



[0034] FIGURE 26 graphically depicts the results of the study carried out in Example 24 wherein percent progressive motility of sperm cells is measured for sperm cells stained with 300 $\mu$ M Hoechst 33342 dye at 41°C in TCA containing 10mM pyruvate or a carbonate-based inhibitor at pH 6.2.

#### Detailed Description of the Preferred Embodiments

[0035] Surprisingly, it has been determined that sperm cells can be stained with a dye for use in flow cytometry processes at elevated temperatures, *i.e.*, at temperatures in excess of 40°C, in less time than is required to stain the cells at lesser temperatures without significant impact upon sperm cell viability. In one embodiment, the sperm cells are exposed to the dye at a temperature of at least 41°C. In another embodiment, the sperm cells are exposed to the dye at a temperature of at least 42°C. In another embodiment, the sperm cells are exposed to the dye at a temperature of at least 43°C. In another embodiment, the sperm cells are exposed to the dye at a temperature of at least 44°C. In another embodiment, the sperm cells are exposed to the dye at a temperature of at least 45°C. In general, however, exposing the sperm cells to a temperature substantially in excess of 45°C for any significant period of time may deleteriously affect the viability of the cells. Thus, in one embodiment, the sperm cells are exposed to the dye at a temperature of at least 41°C but not in excess of 50°C. In another embodiment, the sperm cells are exposed to the dye at a temperature of at least 41°C but not in excess of 48°C. In another embodiment, the sperm cells are exposed to the dye at a temperature of at least 41°C but not in excess of 47°C. For example, in one presently preferred embodiment, the sperm cells are exposed

to the dye at a temperature of at least 42°C but not in excess of 47°C. By way of further example, in another presently preferred embodiment, the sperm cells are exposed to the dye at a temperature of at least 43°C but not in excess of 45°C.

[0036] The process of the present invention may be used to stain intact, viable bovine, porcine, equine, or other mammalian sperm cells, derived from a freshly obtained semen sample or from a thawed cryopreserved semen sample. Various methods of collection of viable sperm are known and include, for example, an artificial vagina method or a gloved hand method. A typical bovine semen sample will contain about 0.5 to about 5 billion sperm cells per milliliter depending upon species and the particular animal within the species.

[0037] In general, sperm cells are stained in accordance with one aspect of the present invention by forming a staining mixture which comprises sperm cells and a DNA selective dye. Sperm cells are somewhat sensitive to significant changes in osmotic pressure and pH. To minimize impact upon sperm viability, therefore, it is generally preferred that the staining mixture be formed with these considerations in mind. Consistent with these considerations, the staining mixture may be formed in a variety of manners.

[0038] In one embodiment, neat semen is combined with the DNA selective dye. Thus, for example, dye in the form of a neat solid, including a free-flowing powder, or a liquid composition may be combined with neat semen. Alternatively, an unbuffered liquid in which the dye is dissolved or dispersed may be combined with neat semen. In each of these approaches, however, it is generally preferred that the formation of the staining mixture not cause the sperm to be subjected to a significant change in

osmotic pressure or pH relative to neat semen sufficient to materially adversely affect their viability.

[0039] In another embodiment, neat semen is combined with a buffered liquid in which the dye is dissolved or  
5 dispersed to form the staining mixture. Advantageously, the buffer will aid in the avoidance of any significant change in osmotic pressure or pH relative to neat semen sufficient to materially adversely affect their viability as a consequence of the formation of the mixture.

10 [0040] In another embodiment, a sperm source is combined with a buffer to form a sperm suspension and the sperm suspension is thereafter combined with the dye to form the staining mixture. Suspending the sperm cells in a buffered liquid prior to contact with the dye can  
15 advantageously protect the sperm cells from significant changes in pH and osmotic pressure which would otherwise result from the addition of the dye. In this embodiment, the sperm source may be neat semen. Alternatively, the sperm source may be a sperm-containing semen derivative  
20 obtained by centrifugation or the use of other means to separate semen into fractions.

[0041] In another embodiment, the DNA selective dye is combined with a buffer, or is included as part of a buffer recipe, thereby forming a buffered dye solution. Thus, for  
25 example, dye in the form of a neat solid, including a free-flowing powder, or a liquid composition may be combined with the buffer, or any constituents of the buffer recipe prior to the combination thereof, to form a buffered dye solution, which may then be combined with neat semen or a  
30 sperm suspension.

[0042] A variety of biological buffers may be used, individually or in combination, to protect the sperm cells from significant changes in pH and osmotic pressure. Typically, these buffers will be in a concentration of

about 0.001M to about 1.0M and at a pH between about 4.5 to about 8.5. Common biological buffers include, for example, phosphates, diphosphates, citrates, acetates, and lactates. Such buffers may also contain a nutrient source, such as

5 for example sugar, and/or an antibiotic, such as streptomycin. Preferred buffers include TCA, TEST, sodium citrate, TL, and HEPES. In certain embodiments of the invention, the semen sample is diluted with one or more of the buffer solutions described in Table I. For example, in

10 one embodiment, the semen sample is diluted with a TCA#1 buffer solution having the composition described in Table I. In another embodiment, the semen sample is diluted with a TCA#2 buffer solution having the composition described in Table I. In another embodiment, the semen sample is

15 diluted with a TEST buffer solution having the composition described in Table I. In another embodiment, the semen sample is diluted with a sodium citrate buffer solution having the composition described in Table I. In yet another embodiment, the semen sample is diluted with a TL

20 buffer solution having the composition described in Table I. In another embodiment, the semen sample is diluted with a HEPES buffer solution having the composition described in Table I. In a further embodiment, the sperm cells may be diluted with a buffer solution comprising 0.204g  $\text{NaHCO}_3$ ,

25 0.433g  $\text{KHCO}_3$ , and 0.473g  $\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$  per 25mL of purified water (0.097 moles/L of  $\text{NaHCO}_3$ , 0.173 moles/L of  $\text{KHCO}_3$ , 0.090 moles/L  $\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$  in water) to form a sperm suspension.

Table I

| BUFFER RECIPES  |         |         |         |            |         |         |
|---|---------|---------|---------|------------|---------|---------|
| COMPONENTS  | TCA#1   | TCA#2   | TEST    | Na Citrate | HEPES   | TL      |
| Sodium chloride (NaCl)  |         |         |         |            | 7.6g    | 5.84g   |
| Potassium chloride (KCl)  |         |         |         |            | 0.3g    | 0.23g   |
| sodium bicarbonate (NaHCO <sub>3</sub> )  |         |         |         |            |         | 2.1g    |
| Sodium phosphate monobasic (NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O) |         |         |         |            |         | 0.04g   |
| (+)-2-hydroxypropionic acid (Na Lactate)  |         |         |         |            |         | 3.68ml  |
| magnesium chloride (MgCl <sub>2</sub> )   |         |         |         |            | 0.1g    | 0.08g   |
| N-(2-hydroxyethyl)piperazine-N'-(2-ethansulfonic acid) (HEPES)                  |         |         |         |            | 2.38g   | 2.38g   |
| tris(hydroxymethyl) amimonethane (TRIS base)                                    | 30.3g   | 32.02g  | 10.28g  |            |         |         |
| Citric Acid Monohydrate   | 15.75g  | 18.68g  |         |            |         |         |
| Na Citrate Dihydrate  |         |         |         | 29g        |         |         |
| 2-[(2-hydroxy-1,1-bis[hydroxymethyl] ethyl) aminoethanesulfonic acid (TES)      |         |         | 43.25g  |            |         |         |
| Fructose  | 12.5g   | 2.67g   |         | 10g        | 2.52g   |         |
| D-Glucose   |         |         | 2g      |            |         |         |
| Streptomycin  |         |         | 0.25g   |            |         |         |
| Penicillin-G  |         |         | 0.15g   |            |         |         |
| Water   | 1 liter | 1 liter | 1 liter | 1 liter    | 1 liter | 1 liter |
| target pH   | 7.35    | 7.35    | 7.35    | 7.35       | 7.35    | 7.35    |
| target osmolality (milliosmols/kg H <sub>2</sub> O)                             | ~314    | ~300    | ~302    | ~316       | ~298    | ~296    |

[0043] The amount of buffer employed generally depends  
 5 upon several considerations, e.g., the particular buffer  
 and the desired sperm concentration (# sperm/ml) in the  
 staining mixture. Therefore, a sufficient amount of buffer  
 will be used such that the desired concentration of  
 sperm/ml is achieved. In one embodiment, buffer is added  
 10 to achieve a sperm suspension that contains less than the  
 concentration of sperm in a neat semen sample. In another  
 embodiment, buffer is added to achieve a sperm suspension  
 that contains from about  $1 \times 10^6$  sperm/ml to about  $5 \times 10^9$   
 sperm/ml. In another embodiment, buffer is added to  
 15 achieve a sperm suspension that contains less than about

200 X 10<sup>6</sup> sperm/ml. In yet another embodiment, buffer is added to achieve a sperm suspension that contains from about 1 X 10<sup>6</sup> sperm/ml to about 200 X 10<sup>6</sup> sperm/ml. In still another embodiment, buffer is added to achieve a sperm suspension that contains from about 20 X 10<sup>6</sup> sperm/ml to about 200 X 10<sup>6</sup>. In another embodiment, buffer is added to achieve a sperm suspension that contains from about 30 X 10<sup>6</sup> sperm/ml to about 175 X 10<sup>6</sup> sperm/ml. In still another embodiment, buffer is added to achieve a sperm suspension that contains from about 50 X 10<sup>6</sup> sperm/ml to about 150 X 10<sup>6</sup>. In yet another embodiment, buffer is added to achieve a sperm suspension that contains from about 100 X 10<sup>6</sup> sperm/ml to about 150 X 10<sup>6</sup> sperm/ml. In still another embodiment, buffer is added to achieve a sperm suspension that contains about 150 X 10<sup>6</sup> sperm/ml.

[0044] The concentration of the dye in the staining mixture is selected to bind a sufficient amount of dye to the DNA to enable X and Y chromosome-bearing sperm to be sorted into gender enriched populations. In addition, the dye concentration is preferably sufficient to provide quantitative staining of the sperm cells in a reasonably short period without meaningfully impacting cell viability. Factors influencing the desired concentration for a particular application include the nature of the dye, the concentration of sperm in the staining mixture, the pH of the staining mixture, the length of time permitted for uptake of the dye by the sperm cells, and the temperature during this uptake period. In general, however, the concentration of dye in the staining mixture will typically be at any of a range of concentrations, generally between about 0.1 $\mu$ M and about 1000 $\mu$ M. For example, the concentration of the dye may be maintained at a "relatively low" concentration range, i.e., a concentration of about 0.1 $\mu$ M to about 250 $\mu$ M; within this embodiment, the

concentration is preferably from about 100 $\mu$ M to about 200 $\mu$ M, more preferably about 100 $\mu$ M, still more preferably about 200 $\mu$ M, and even still more preferably about 150 $\mu$ M. Alternatively, the concentration of the dye may be

5 maintained within an "intermediate" concentration range, i.e., a concentration of about 250 $\mu$ M to about 700 $\mu$ M; within this embodiment, the concentration is preferably from about 300 $\mu$ M to about 700 $\mu$ M, more preferably about 300 $\mu$ M, still more preferably about 400 $\mu$ M, and even still more preferably

10 about 600 $\mu$ M. In addition, the concentration of the dye may be maintained within a "relatively high" concentration range, i.e., a concentration of about 700 $\mu$ M to about 1000 $\mu$ M; within this embodiment, the concentration is preferably from about 800 $\mu$ M to about 1000 $\mu$ M, more

15 preferably about 800 $\mu$ M, still more preferably about 900 $\mu$ M, and even still more preferably about 1000 $\mu$ M. Accordingly, in one embodiment, the dye concentration is about 100 $\mu$ M to about 200 $\mu$ M. In another embodiment, the dye concentration is about 10 $\mu$ M to about 100 $\mu$ M. In another embodiment, the

20 dye concentration is about 20 $\mu$ M to about 80 $\mu$ M. In still another embodiment, the dye concentration in the staining mixture is about 20 $\mu$ M to about 60 $\mu$ M. In addition, it has been reported that the optimal concentration of stain for most species has been reported to be about 40 $\mu$ g per 150 x

25 10<sup>6</sup> sperm, which is approximately 70 $\mu$ M. See, for example, L. A. Johnson and Glenn Welch, *Theriogenology* (1999).

[0045] The pH of the staining mixture is preferably maintained in the range of about 6.0 to about 8.0. More preferably, the pH of the staining mixture is maintained in

30 the range of about 7.1 to about 7.6. Still more preferably, the pH of the staining mixture is maintained at about 7.3 to 7.4. Still more preferably, the pH of the staining mixture is maintained at about 7.35.

[0046] Certain dyes are capable of permeating the sperm cells and specifically binding the DNA without further intervention to increase the permeability of the cells. With other dyes, however, it may be desirable to  
5 treat the sperm prior to staining to increase the rate of permeation without unacceptably reducing viability or motility. Any suitable method known to those skilled in the art may be used. Such methods include electroporation, the use of cell-permeation-enhancing solutions, e.g., mild  
10 surfactants, or chemical shock. Where it is desired or advantageous to use other or more stringent techniques, such treatments can include the use of liposomes or many of the techniques which are used by those skilled in the art to introduce stains, dyes, genes, or vectors into living  
15 cells. These methods include, but are not limited to microinjection such as used by Gordon et al. (*Proc. Natl. Acad. Sci.*, 1980) and since extended to rabbits, sheep, cattle and pigs; DEAE-dextran-mediated transfer; coprecipitation with calcium phosphate; and other  
20 techniques, all of which are well known to one of skill in the art. In yet other instances, it may be desirable to centrifuge the sperm and re-suspend the centrifuged sperm in another medium, albeit based on the same or substantially the same buffer system to remove certain  
25 components (which may have previously been added to the sperm suspension) that may interfere with later processing steps.

[0047] Uptake of dye by the sperm cells in the staining mixture is allowed to continue for a period of  
30 time sufficient to obtain the desired degree of DNA staining. In general, the uptake period will be between about 1 and about 160 minutes. In one embodiment, the uptake period is less than 90 minutes. In another embodiment, the uptake period is less than 60 minutes. In



another embodiment, the uptake period is less than 40 minutes. In another embodiment, the uptake period is less than 25 minutes. As previously noted, the uptake period is somewhat dependent upon a range of other parameters, including the uptake temperature, the nature of the dye and the concentration of the dye. In certain embodiments, the uptake period is about 2 to about 25 minutes. In other embodiments, the uptake period is about 5 to 20 minutes. In still other embodiments, the uptake period is about 2 to about 10 minutes. In another embodiment, the uptake period is less than about 2 minutes.

[0048] The staining mixture may be subjected to an elevated temperature of the present invention for the entire uptake period or for a fraction thereof. Thus, for example, the staining mixture may be maintained at a temperature in excess of 40°C for at least 1%, 5%, 10%, 20% or even a greater percentage of the uptake period. In one embodiment, the temperature of the staining mixture is increased during the uptake period. In another embodiment, the temperature of the staining mixture is decreased during the uptake period. In each of these embodiments, however, the rate of temperature change is controlled to preferably avoid any significant negative impact upon sperm cell viability.

[0049] In any event, the uptake period is sufficient for the dye to bind to the DNA such that X and Y chromosome-bearing sperm cells can be sorted based upon the differing and measurable fluorescence intensity between the X and Y chromosome-bearing sperm. In one embodiment, the degree of staining is sufficient to permit the X and Y chromosome bearing sperm to be differentially sorted based upon their respective fluorescence into X and Y populations of at least about 60% purity. The degree of staining is preferably sufficient to permit the X and Y chromosome

bearing sperm to be differentially sorted based upon their respective fluorescence into X and Y chromosome bearing sperm populations of at least about 70% purity, more preferably at least about 80% purity, even more preferably at least about 85% purity, and still more preferably at least about 90% purity.

[0050] Upon being bound to the DNA and excitation with UV or visible light, the dyes of the present invention fluoresce. In one embodiment, the dye is one which fluoresces upon excitation with ultraviolet radiation. Such dyes include, for example, bisbenzimidides such as Hoechst 33342 and Hoechst 33258, each of which is commercially available from Sigma-Aldrich (St. Louis, MO). Advantageously, for example, Hoechst 33342 has a low toxicity, is sufficiently cell permeable, is specific for DNA, has a fluorescence that is dramatically enhanced after binding to DNA, and displays a linear relationship between the intensity of the fluorescence and the amount of DNA present in a given cell or sample.

[0051] In another embodiment, the dye is one which fluoresces upon excitation with visible light. Such dyes include, for example, the visible light excitable dye, SYBR-14, commercially available from Molecular Probes, Inc. (Eugene, OR) and the bisbenzimidide-BODIPY® conjugate 6-{[3-((2Z)-2-{[1-(difluoroboryl)-3,5-dimethyl-1H-pyrrol-2-yl]methylene}-2H-pyrrol-5-yl)propanoyl]amino}-N-[3-(methyl{3-[(4-[6-(4-methylpiperazin-1-yl)-1H,3'H-2,5'-bibenzimidazol-2'-yl]phenoxy}acetyl)amino]propyl]amino)propyl]hexanamide ("BBC") described in WO 02/41906.

[0052] In one embodiment of the present invention, the dye may be modified by conjugation to another moiety. For example, in accordance with a particular aspect of the invention, the bisbenzimidide (bisbenzimidazole) can be

modified by addition of a fluorophore that results in a fluorescence response by the conjugate to excitation by visible light. Preferably these conjugate molecules resemble the bisbenzimidazole molecule in that binding to DNA enhances their fluorescence, and represent an improvement over the bisbenzimidazole molecule in that the conjugates fluoresce in response to visible light

[0053] Particularly preferred fluorophores are visible-light-excitable dipyrrometheneboron difluoride derivatives. Dipyrrometheneboron difluoride dyes are membrane permeant fluorescent compounds available from Molecular Probes Inc. under the BODIPYO trademark as described in, for example, US Patent Nos. 5,338,854 and 4,774,339, herein incorporated by reference. Preparation of an exemplary bisbenzimidazole-dipyrrometheneboron difluoride conjugate is described WO 02/41906. Other fluorophores of the class described in the preceding paragraph, such as, for example, fluorescein and its derivatives may also be used.

[0054] Those skilled in the art will appreciate that such fluorophore-modified dyes, may be prepared by modifying or functionalizing the conjugate DNA stains with otherwise suitable properties so that they have sufficient cell permeability in the desired pH and temperature ranges. For example, chemical modifications can be made to enhance appropriate cell permeability by (1) changing the pKa of the DNA dye, (2) adding an ionic permeability-enhancing group, either cationic or anionic, attached through an appropriate linker, or (3) adding nonionic permeability-enhancing groups such as ethylene glycol or polyethylene glycol moieties.

[0055] Within the scope of the invention, the bisbenzimidazole and visible wavelength fluorophore can be connected in many different ways. WO 02/41906 illustrates

one way they can be connected; however, persons skilled in the art can readily select many other fluorophores and methods of connection. Supplies and consultation services to assist in such selection are readily available to those  
5 skilled in the art from commercial entities in the business of making and selling the fluorophores such as, for example, Molecular Probes Inc., (Eugene, OR).

[0056] Preferably, the chemical entity linking the bisbenzimidazole to the visible wavelength fluorophore will be  
10 selected to not result in significant negative effects upon viability, permeability, stability, uptake, cell storage, flow cytometry, formulation, or fluorescence properties. Preferably the chemical functionality of the linking entity will be selected to enhance properties such as stability,  
15 permeability, viability, uptake, cell storage, flow cytometry, formulation, or fluorescence properties.

[0057] The methods disclosed herein may be applied either to sperm recently obtained from the particular source (i.e., obtained from bovine, porcine, or other  
20 mammalian source within minutes or hours before staining) or to sperm that have been cryopreserved and subsequently thawed. In either instance, the results of the cytometric sorting may be improved by the addition of a quencher to the staining solution to reduce the fluorescence of dead  
25 sperm. Various quenchers, as well as the use of the same, are well known in the art, as is demonstrated with respect to propidium iodide (Garner et al., *Bio. of Reprod.*, 53: 276-84 (1995)) and FD&C #40 (Johnson et al.,  
*Theriogenology*, 52: 1323-1341 (1999)). FD&C #40 is  
30 especially useful in this method, as it is nontoxic if used in low concentrations. Likewise, the combination of SYBR-14 and propidium iodide is advantageous, as this allows visualization and separation of live sperm cells from moribund and dead cells. Accordingly, in one embodiment of

the invention, the sperm cells are combined with both a dye and a quencher to form a staining mixture. In a preferred embodiment, the sperm cells are combined with Hoechst 33342 and FD&C #40. In yet another embodiment, the sperm cells  
5 are combined with SYBR-14 and propidium iodide. While quenchers are useful when sorting thawed cryopreserved sperm samples, it is to be understood that their use is not solely limited to such, and that they may be used advantageously on samples of recently obtained sperm.

10       [0058] In addition to buffer, other additives may be included in the staining mixture to enhance the viability or motility of the sperm; these additives may be provided as part of the sperm source, the dye source, or separately to the staining mixture. Such additives include energy  
15 sources, antibiotics, compositions which regulate oxidation/reduction reactions intracellularly or extracellularly, motility inhibitors, and seminal plasma.

          [0059] In general, motility inhibitors cause the cells in the staining mixture to emulate sperm cells of the  
20 epididymis of a mammal, such as for example a bull, by simulating the fluid environment of the epididymis or epididymal tract of the mammal; thus for example, the inhibitor(s) inhibit the metabolic activity and/or the motility of the sperm. The inhibitor may be any of a range  
25 of compositions having a depressive effect upon sperm motility. For example, relatively high concentrations of potassium ions in the staining mixture tend to depress sperm motility. In one embodiment, therefore, it is preferred that the staining mixture contain a source of  
30 potassium ions and that the potassium concentration in the staining mixture be at least about 0.05 moles/L. More preferably, the potassium concentration is at least about 0.05 moles/L to about 0.5 moles/L. Still more preferably, the potassium concentration is at least about 0.1 moles/L

to about 0.3 moles/L. Most preferably, the potassium concentration is at about 0.173 moles/L. Such staining mixtures will typically, but not necessarily, also contain a source of sodium ions. When sodium is present, the molar ratio of potassium to sodium is greater than 1:1, respectively. Preferably, the molar ratio of potassium to sodium is at least about 1.25:1. Still more preferably, the molar ratio of potassium to sodium is at least about 1.5:1. Still more preferably, the molar ratio of potassium to sodium is at least about 1.75:1. Still more preferably, the molar ratio of potassium to sodium is at least about 1.78:1. In one particular embodiment, the molar ratio of potassium to sodium is at least about 2:1.

[0060] The staining mixture may additionally comprise an ion or source of carbon dioxide capable of down-regulating uptake of carbohydrate. In this embodiment, the source of carbon dioxide may be, for example, one or more carbonates. In one embodiment, the staining mixture comprises  $\text{NaHCO}_3$  and  $\text{KHCO}_3$ , thereby providing a source of potassium and sodium ions as well as a partial pressure of carbon dioxide. For example, in one embodiment, the staining mixture comprises  $\text{NaHCO}_3$  and  $\text{KHCO}_3$  in an aqueous solution, preferably  $\text{NaHCO}_3$ ,  $\text{KHCO}_3$ , and  $\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$  in water; by way of further example, the staining mixture may be formed using an inhibitory buffer comprising 0.097 moles/L of  $\text{NaHCO}_3$ , 0.173 moles/L of  $\text{KHCO}_3$ , 0.090 moles/L  $\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$  in water as disclosed in Salisbury & Graves, *J. Reprod. Fertil.*, 6:351-359 (1963). The sperm cells will generally remain quiescent as long as they are exposed to the motility inhibitor(s).

[0061] Examples of such a composition which regulates oxidation/reduction reactions intracellularly or extracellularly include, for example, pyruvate, vitamin K, lipoic acid, glutathione, flavins, quinones, superoxide

dismutase (SOD), and SOD mimics. If included in the staining mixture, such a composition may be present in a concentration sufficient to effect the protective effect without detrimentally affecting sperm health. Exemplary

5 concentration ranges include from about 10 $\mu$ M to about 50mM depending upon such factors as the particular composition being used or the concentration of sperm in the staining mixture. For example, if pyruvate is included in the composition, it may be present in the staining mixture in a

10 concentration from about 0.5 $\mu$ M to about 50mM, preferably from about 1mM to about 40mM, more preferably from about 2.5mM to about 25mM, still more preferably from about 10mM to about 20mM, even still more preferably at about 15mM, and most preferably at about 10mM. If vitamin K is

15 included in the composition, it may be present in the staining mixture in a concentration from about 1 $\mu$ M to about 100 $\mu$ M, preferably from about 10 $\mu$ M to about 100 $\mu$ M, more preferably from about 50 $\mu$ M to about 100 $\mu$ M, and most preferably at about 100 $\mu$ M. If lipoic acid is included in

20 the composition, it may be present in the staining mixture in a concentration from about 0.1mM to about 1mM, preferably from about 0.5mM to about 1mM, more preferably about 0.5mM, and most preferably about 1mM. The staining mixture may comprise any one of the above listed

25 embodiments of the composition or any combination thereof in the above listed concentrations. For example, the staining mixture may comprise a composition which regulates oxidation/reduction reactions intracellularly or extracellularly comprising pyruvate in a concentration of

30 about 10mM and vitamin K in a concentration of about 100 $\mu$ M. Alternatively, the staining mixture may comprise a composition comprising pyruvate in a concentration of about 10mM and lipoic acid in a concentration of about 1mM. Yet

another example includes a staining mixture comprising a composition comprising pyruvate in a concentration of about 10mM, vitamin K in a concentration of about 100µM, and lipoic acid in a concentration of about 1mM.

5           [0062] Once the sperm are stained according to the present invention, they may be prepared for sorting and then sorted according to any known means that allows for separation based upon fluorescence. Commonly used and well known methods include flow cytometry systems, as  
10 exemplified by and described in U.S. Patent Nos. 5,135,759, 5,985,216, 6,071,689, 6,149,867, and 6,263,745, as well as WO 99/33956 and WO 01/37655. In one embodiment, for example, the stained cells may be combined with a sheath fluid or otherwise prepared for sorting while being  
15 maintained at an elevated temperature of the present invention. In another embodiment, for example, the stained cells are cooled from the elevated temperature at which dye uptake occurs to a lesser temperature, e.g., room temperature, at which later processing steps are to be  
20 carried out; to enable the desired degree of separation in this embodiment, however, it is generally preferred that no significant amount of dye uptake occurs at temperatures less than the elevated temperatures of the present invention.

25

### Examples

#### Example 1.

          [0063] Bull semen was collected from a sexually mature bull using an artificial vagina and the sample transported  
30 at 37°C in a temperature-controlled container to the staining facility. Upon receipt, the semen was analyzed for concentration, visual motility, pH, and membrane integrity, motility and progressive motility by the Hamilton-Thorn Motility Analyzer (IVOS), according to a



standard and well known procedures (Farrell et al. *Theriogenology*, 49(4): 871-9 (Mar 1998)). Based on the semen concentration, 8 X 5mL sperm suspensions were prepared. Four samples of 5mL of  $150 \times 10^6$  sperm/mL were prepared by suspending an aliquot of semen in 41°C TCA buffer pH 7.35. Four additional samples of 5mL of  $150 \times 10^6$  sperm/mL were prepared by suspending an aliquot of semen in 39°C TCA buffer at pH 7.35. To the sperm suspensions, aliquots of a 10 mM Hoechst solution in water were added to yield the dye concentrations as seen in Table 1 ("Target Concentration of Hoechst ( $\mu$ M)"). The sperm suspensions were maintained in 41°C and 39°C water baths. Sperm suspensions were analyzed by removing an aliquot of 500 $\mu$ L from sperm suspension samples and analyzing by flow cytometry to measure the uptake of the dye (i.e., to determine the Fluorescence Intensity).

Table 1

| Tube # | Temperature (°C) | Target concentration of Hoechst ( $\mu$ M) | $\mu$ L 10mM Hoechst 33342 to be added to 5mL of sperm suspension |
|--------|------------------|--|---|
| 1      | 39°C             | 20 $\mu$ M                                 | 10 $\mu$ L  |
| 2      | 41°C             | 20 $\mu$ M                                 | 10 $\mu$ L  |
| 3      | 39°C             | 30 $\mu$ M                                 | 15 $\mu$ L  |
| 4      | 41°C             | 30 $\mu$ M                                 | 15 $\mu$ L  |
| 5      | 39°C             | 57 $\mu$ M                                 | 28.5 $\mu$ L  |
| 6      | 41°C             | 57 $\mu$ M                                 | 28.5 $\mu$ L  |
| 7      | 39°C             | 85 $\mu$ M                                 | 42.5 $\mu$ L  |
| 8      | 41°C             | 85 $\mu$ M                                 | 42.5 $\mu$ L  |

[0064] Results of the analysis are summarized in Figures 1A-1D.

#### Example 2

[0065] Sperm samples were obtained and prepared in the same manner as in Example 1 with the exception of the dye

concentrations of Hoechst 33342 used to stain the sperm. Table 2 lists the concentrations used in Example 2. The suspensions were maintained in 41°C and 39°C water baths. Sample aliquots of 500µL were removed periodically from  
5 each sample and analyzed by flow cytometry to determine the Fluorescence Intensity.

Table 2

| Tube # | Temperature (°C) | Target concentration of Hoechst (μM) | μL 10mM Hoechst 33342 to be added to 5mL of sperm suspension |
|--------|------------------|--------------------------------------|--|
| 1      | 39°C             | 20μM                                 | 10μL   |
| 2      | 41°C             | 20μM                                 | 10μL   |
| 3      | 39°C             | 30μM                                 | 15μL   |
| 4      | 41°C             | 30μM                                 | 15μL   |
| 5      | 39°C             | 40μM                                 | 20μL   |
| 6      | 41°C             | 40μM                                 | 20μL   |
| 7      | 39°C             | 60μM                                 | 30μL   |
| 8      | 41°C             | 60μM                                 | 30μL   |

[0066] Results of the analysis are summarized in  
5 Figures 2A-2D.

### Example 3

[0067] Sperm samples were obtained, prepared, stained,  
and analyzed in the same manner as in Example 1 with the  
10 exception of the dye concentrations of Hoechst 33342 used  
to stain the sperm and the staining temperatures. Table 3  
lists the concentrations and temperatures used in Example  
3.

Table 3

| Tube # | Temperature (°C) | Target concentration of Hoechst (μM) | μL 10mM Hoechst 33342 to be added to 5mL of sperm suspension |
|--------|------------------|--------------------------------------|--|
| 1      | 39°C             | 60μM                                 | 30μL   |
| 2      | 43°C             | 60μM                                 | 30μL   |
| 3      | 39°C             | 40μM                                 | 20μL   |
| 4      | 43°C             | 40μM                                 | 20μL   |
| 5      | 39°C             | 30μM                                 | 15μL   |
| 6      | 43°C             | 30μM                                 | 15μL   |
| 7      | 39°C             | 20μM                                 | 10μL   |
| 8      | 43°C             | 20μM                                 | 10μL   |

[0068] Results of the analysis are summarized in Figures 3A-3D.

#### 5 Example 4

[0069] Sperm samples were obtained, prepared, stained, and analyzed in the same manner as in Example 1 with the exception of the dye concentrations of Hoechst 33342 used to stain the sperm and the staining temperatures. Table 4  
 10 lists the concentrations and temperatures used in Example 4.

Table 4

| Tube # | Temperature (°C) | Target concentration of Hoechst (μM) | μL 10mM Hoechst 33342 to be added to 5mL of sperm suspension |
|--------|------------------|--------------------------------------|--|
| 1      | 39°C             | 80μM                                 | 40μL   |
| 2      | 43°C             | 80μM                                 | 40μL   |
| 3      | 39°C             | 100μM                                | 50μL   |
| 4      | 43°C             | 100μM                                | 50μL   |

15 [0070] Results of the analysis are summarized in Figures 4A and 4B.

**Example 5**

[0071] Sperm samples were obtained, prepared, stained, and analyzed in the same manner as in Example 1 with the exception of the dye concentrations of Hoechst 33342 used to stain the sperm and the staining temperatures. Table 5 lists the concentrations and temperatures used in Example 5.

**Table 5**

| Tube # | Temperature (°C) | Target concentration of Hoechst (μM) | μL 10mM Hoechst 33342 to be added to 5mL of sperm suspension |
|--------|------------------|--------------------------------------|--|
| 5      | 39°C             | 80μM                                 | 40μL   |
| 6      | 45°C             | 80μM                                 | 40μL   |
| 7      | 39°C             | 100μM                                | 50μL   |
| 8      | 45°C             | 100μM                                | 50μL   |

10

[0072] Results of the analysis are summarized in Figures 5A and 5B.

**Example 6**

15 [0073] Sperm samples were obtained, prepared, stained, and analyzed in the same manner as in Example 1 with the exception of the dye concentrations of Hoechst 33342 used to stain the sperm and the staining temperatures. Table 6 lists the concentrations and temperatures used in Example 20 6.

Table 6

| Tube # | Temperature (°C) | Target concentration of Hoechst ( $\mu\text{M}$ ) | $\mu\text{L}$ 10mM Hoechst 33342 to be added to 5mL of sperm suspension |
|--------|------------------|---|---|
| 1      | 39°C             | 60 $\mu\text{M}$                                  | 30 $\mu\text{L}$  |
| 2      | 47°C             | 60 $\mu\text{M}$                                  | 30 $\mu\text{L}$  |
| 3      | 39°C             | 150 $\mu\text{M}$                                 | 75 $\mu\text{L}$  |
| 4      | 43°C             | 150 $\mu\text{M}$                                 | 75 $\mu\text{L}$  |

[0074] Results of the analysis are summarized in Figures 6A and 6B.

5

**Example 7**

[0075] Bull semen was collected from a sexually mature bull using an artificial vagina and the sample transported at 25°C in a temperature-controlled container to the staining facility. Upon receipt, the semen was analyzed for concentration, visual motility, IVOS motility and progressive motility, pH, and membrane integrity by known analytical methods. Based on the semen concentration, 4 X 1mL of  $150 \times 10^6$  sperm/mL sperm suspensions were prepared. Two samples of 1mL of  $150 \times 10^6$  sperm/mL were prepared by suspending an aliquot of semen in 41°C TCA buffer at pH 7.35. Two additional samples of 1mL of  $150 \times 10^6$  sperm/mL were prepared by suspending an aliquot of semen in 43°C TCA buffer at pH 7.35. To one sample of each temperature was added 6 $\mu\text{L}$  of 10 mM Hoechst solution to yield the dye concentration of 60 $\mu\text{M}$ . The suspensions were maintained in 41°C and 43°C water baths. Periodically 50 $\mu\text{L}$  aliquots were removed from the sperm suspension samples, transferred to a conical tube and 200 $\mu\text{L}$  of the appropriate temperature TCA buffer added, to yield a final sperm suspension concentration of  $30 \times 10^6$  sperm/mL. The  $30 \times 10^6$  sperm/mL sperm suspension samples were immediately analyzed by IVOS.

IVOS results for % Motility and % Progressive Motility (Prog Mot) are shown in Figures 7A and 7B.

#### Example 8

5        [0076] Bull semen was collected, analyzed, suspended in buffer, stained with Hoechst 33342, and analyzed by IVOS as in Example 7 with the following exception. Samples were stained at a concentration of 60 $\mu$ M and maintained at a temperature of 39°C and 45°C. Results of the IVOS analysis  
10 are shown in Figures 8A and 8B.

#### Example 9

      [0077] Bull semen was collected, analyzed, suspended in buffer, stained with Hoechst 33342, and analyzed by IVOS  
15 as in Example 7 with the following exception. Samples were stained at a concentration of 80 $\mu$ M and maintained at temperatures of 41°C, 43°C, and 45°C. Results of the IVOS analysis are shown in Figures 9A-9C.

#### 20 Example 10

      [0078] Bull semen was collected, analyzed, suspended in buffer, stained with Hoechst 33342, and analyzed by IVOS as in Example 7 with the following exception. Samples were stained at a concentration of 100 $\mu$ M and maintained at  
25 temperatures of 41°C, 43°C, and 45°C. Results of the IVOS analysis are shown in Figures 10A-10C.

#### Example 11

      [0079] Bull semen was collected, analyzed, suspended  
30 in buffer, stained with Hoechst 33342, and analyzed by IVOS as in Example 7 with the following exception. Samples were stained at a concentration of 100 $\mu$ M and maintained at a

temperature of 47°C. Results of the IVOS analysis are shown in Figure 11.

#### Example 12

5        [0080] Bull semen was collected, analyzed, suspended in buffer, stained with Hoechst 33342, and analyzed by IVOS as in Example 7 with the following exception. Samples were stained at a concentration of 100µM and maintained at a temperature of 49°C. Results of the IVOS analysis are shown  
10 in Figure 12.

#### Example 13

      [0081] Bull semen was collected, analyzed, suspended in buffer, stained with Hoechst 33342, and analyzed by IVOS  
15 as in Example 7 with the following exception. Samples were stained at a concentration of 125µM and 150µM, and each was maintained at a temperature of 43°C in a water bath. Results of the IVOS analysis are shown in Figures 13A and  
20 13B.

#### Example 14

      [0082] Bull semen was collected, analyzed, suspended in buffer, stained with Hoechst 33342, and analyzed by IVOS  
25 as in Example 7 with the following exception. Samples were stained at a concentration of 200µM and 250µM, and each was maintained at a temperature of 43°C in a water bath. Results of the IVOS analysis are shown in Figures 14A and  
14B.

#### 30 Example 15

      [0083] Bull semen was collected, analyzed, suspended in buffer, stained with Hoechst 33342, and analyzed by IVOS as in Example 7 with the following exception. Samples were



stained at a concentration of 125 $\mu$ M and 150 $\mu$ M, and each was maintained at a temperature of 45°C in a water bath. Results of the IVOS analysis are shown in Figures 15A and 15B.

5

**Example 16**

[0084] Bull semen was collected, analyzed, suspended in buffer, stained with Hoechst 33342, and analyzed by IVOS as in Example 7 with the following exception. Samples were  
10 stained at a concentration of 200 $\mu$ M, 250 $\mu$ M, and 300 $\mu$ M, and each was maintained at a temperature of 45°C in a water bath. Results of the IVOS analysis are shown in Figures 16A-16C.

15 **Example 17**

[0085] Bull semen was collected, analyzed, suspended in buffer, stained with Hoechst 33342, and analyzed by IVOS as in Example 7 with the following exception. Samples were stained at a concentration rate of 300 $\mu$ M, 350 $\mu$ M, and 400 $\mu$ M,  
20 and each was maintained at a temperature of 43°C in a water bath. Results of the IVOS analysis are shown in Figures 17A-17C.

**Example 18**

25 [0086] Bull semen was collected from a sexually mature bull using an artificial vagina and the sample transported at 25°C in a temperature-controlled container to the staining facility. Upon receipt, the semen was analyzed for concentration, visual motility, IVOS motility and  
30 progressive motility, pH, and membrane integrity by known analytical methods. Based on the semen concentration, 4 X 1mL of 150 X 10<sup>6</sup> sperm/mL sperm suspensions were prepared by suspending aliquots of semen in 43°C TCA buffer at pH 7.35.

To three of the samples was added SYBR 14 dye solution to yield the dye concentrations of 0.6 $\mu$ M, 6 $\mu$ M, and 60 $\mu$ M. The suspensions were maintained in a 43°C water bath.

Periodically sample aliquots of 50 $\mu$ L were transferred to a  
5 conical tube and 200 $\mu$ L of the appropriate temperature TCA  
buffer added, to yield a final sperm concentration of 30 X  
10<sup>6</sup> sperm/mL. The samples were immediately analyzed on the  
IVOS. IVOS results for % Motility and % Progressive  
Motility (Prog Mot) are shown in Figures 18A-18C.

10

#### **Example 19**

[0087] Sperm samples were obtained, prepared, stained,  
and analyzed in the same manner as Example 1 with the  
exception of the dye and concentrations used. BBC,  
15 concentration 100 $\mu$ M, and SYBR 14, concentration 6 $\mu$ M, were  
used to stain the sperm suspensions and the temperatures  
maintained @ 45°C in a water bath. Table 7 lists a summary  
of the concentrations and temperatures used in Example 19.

Table 7

| Tube # | Temperature (°C) | Target concentration of SYBR 14 (μM) | μL 1mM SYBR 14 to be added to 5mL of sperm suspension |
|--------|------------------|--------------------------------------|---|
| 1      | 39°C             | 6μM                                  | 30μL  |
| 2      | 45°C             | 6μM                                  | 30μL  |
|        |                  | BBC                                  | μL 10mM BBC   |
| 3      | 39°C             | 100μM                                | 50μL  |
| 4      | 45°C             | 100μM                                | 50μL  |

[0088] Results of the analysis are summarized in the  
 5 Figures 19A and 19B

#### Example 20

[0089] Bull semen was collected from a sexually mature bull using an artificial vagina and the sample diluted in 2  
 10 parts carbonate buffer for transportation at 25°C in a temperature-controlled container to the staining facility. Upon receipt, the semen was analyzed for concentration, motility and progressive motility by the Hamilton-Thorn Motility Analyzer (IVOS), according to standard and well  
 15 known procedures (Farrell et al. *Theriogenology*, 49(4): 871-9 (Mar 1998)). Based on the semen concentration, 1mL of  $150 \times 10^6$  sperm/ml suspension was prepared by removing an aliquot of the carbonate sperm suspension centrifuging the sperm suspension at  $500 \times g$  for 5 minutes, removing the  
 20 supernatant and re-suspending the pellet in 41°C TCA buffer pH 7.3. An additional 1mL of  $150 \times 10^6$  sperm/ml was prepared by suspending an aliquot of semen in 41°C TCA buffer containing 10mM pyruvate at pH 7.3. To the sperm suspensions, aliquots of a 10mM Hoechst solution in water  
 25 were added to yield the dye concentration of 400μM Hoechst. The sperm suspensions were maintained in a 41°C water bath

for the duration of the staining period. Sperm suspensions were analyzed by removing a 50 $\mu$ L aliquot from the staining sperm suspension, adding 200 $\mu$ L of the same buffer at the same temperature and analyzing by IVOS to measure % progressive motility (% Prog Mot). Results of the IVOS analysis are summarized in Figure 20.

#### Example 21

[0090] Sperm samples were obtained and prepared in the same manner as in Example 20 with the following exception. The buffer used to suspend the sperm for staining and IVOS analysis were TCA and TCA containing 10 $\mu$ M Vitamin K. Results of the IVOS analysis are summarized in Figure 21

#### Example 22

[0091] Sperm samples were obtained and prepared in the same manner as in Example 20 with the following exception. The buffer used to suspend the sperm for staining and IVOS analysis were TCA and TCA containing 100 $\mu$ M Vitamin K. Results of the IVOS analysis are summarized in Figure 22.

#### Example 23

[0092] Sperm samples were obtained and prepared in the same manner as in Example 20 with the following exception. The buffers used to suspend the sperm for staining and IVOS analysis were TCA and TCA containing 1mM Lipoic Acid. Results of the IVOS analysis are summarized in Figure 23.

#### Example 24

[0093] Bull semen was collected from a sexually mature bull using an artificial vagina and transported at 25°C in a temperature-controlled container to the staining facility. Upon receipt, the semen was analyzed for concentration, motility and progressive motility by the Hamilton-Thorn

Motility Analyzer (IVOS), according to standard and well known procedures (Farrell et al. Theriogenology, 49(4): 871-9 (Mar 1998)). Based on the semen concentration, several tubes of  $450 \times 10^6$  sperm/ml suspensions were prepared by suspending semen in either a TCA buffer or a carbonate based inhibitor. Table II. below illustrates the compositions and staining conditions used.

Table III

| Sample Name   | Buffer                            | pH  | Conc (uM) Hoechst | Temperature (°C) |
|---------------|-----------------------------------|-----|-------------------|------------------|
| 10mM pyr TCA  | 10mM pyruvate in TCA              | 7.3 | 300 $\mu$ M       | 41°C             |
| Carbonate 6.2 | Carbonate based inhibitor, pH 6.2 | 6.2 | 300 $\mu$ M       | 41°C             |
| Carbonate 7.3 | Carbonate based inhibitor, pH 7.3 | 7.3 | 300 $\mu$ M       | 41°C             |

10

[0094] To the sperm suspensions, aliquots of a 10mM Hoechst solution in water were added to yield a concentration of 300 $\mu$ M Hoechst. The sperm suspensions were maintained in a 41°C water bath for 30 minutes, and then diluted to  $150 \times 10^6$  sperm/ml with 10 mM pyruvate in TCA or a carbonate-based inhibitor at pH 6.2 as specifically indicated in each figure to dilute to a concentration typical for sorting. Sperm suspensions were analyzed by removing a 50 $\mu$ L aliquot from the stained and diluted sperm suspension at the time period designated within each figure and adding 200 $\mu$ L of 25°C 10mM pyruvate in TCA at pH 7.3 to initiate the reversal of the quiescence, allowing at least a five minute equilibration period, and analyzing by IVOS to measure the percent progressive motility. Comparisons of the IVOS percent progressive motilities are seen in Figures 24-26.

15

20

25

**What is Claimed is:**

1. A process for staining sperm cells, the process comprising forming a staining mixture containing intact viable sperm cells and a DNA selective fluorescent dye, and subjecting the staining mixture to a temperature in excess of  
5 40°C.
2. The process of claim 1, wherein the dye is a UV excitable or a visible light excitable dye.
3. The process of claim 2, wherein the dye is selected from the group consisting of a bisbenzimidazole, SYBR-14, and a conjugate, an analog, or a derivative thereof.
4. The process of claim 3, wherein the dye is selected from the group consisting of Hoechst 33342, Hoechst 33258, SYBR-14, and 6-{[3-((2Z)-2-{[1-(difluoroboryl)-3,5-dimethyl-1H-pyrrol-2-yl]methylene}-2H-pyrrol-5-yl)propanoyl]amino}-N-[3-  
5 (methyl{3-[(4-{6-(4-methylpiperazin-1-yl)-1H,3'H-2,5'-bibenzimidazol-2'-yl]phenoxy}acetyl)amino]propyl}amino)propyl]hexanamide.
5. The process of claims 1, wherein the staining mixture is subjected to the temperature for a period of time sufficient to allow the dye to bind the DNA such that X and Y bearing sperm cells can be differentially sorted based upon  
5 fluorescence.
6. The process of claim 5, wherein the period of time is from about 1 minute to about 160 minutes.
7. The process of claim 5, wherein the period of time is less than about 60 minutes.

8. The process of claim 5, wherein the period of time is less than about 30 minutes.
9. The process of claim 5, wherein the dye concentration is from about  $0.1\mu\text{M}$  to about  $1000\mu\text{M}$ .
10. The process of claim 9, wherein the dye concentration is from about  $100\mu\text{M}$  to about  $600\mu\text{M}$ .
11. The process of claim 5, wherein the staining mixture is subjected to a temperature in excess of about  $41^{\circ}\text{C}$ .
12. The process of claim 11, wherein the staining mixture is subjected to a temperature of between about  $41^{\circ}\text{C}$  and about  $50^{\circ}\text{C}$ .
13. The process of claim 12, wherein the staining mixture is subjected to a temperature of between about  $41^{\circ}\text{C}$  and about  $47^{\circ}\text{C}$ .
14. The process of claim 13, wherein the staining mixture is subjected to a temperature of between about  $42^{\circ}\text{C}$  and about  $45^{\circ}\text{C}$ .
15. The process of claim 14, wherein the staining mixture is subjected to a temperature of about  $43^{\circ}\text{C}$ .
16. The process of claim 1, wherein the step of forming a staining mixture comprises combining a buffer with the sperm cells.
17. The process of claim 16, wherein the buffer is combined with the sperm cells to form a sperm suspension, and the sperm

suspension is combined with a DNA selective dye to form the staining mixture.

18. The process of claim 1, wherein the step of forming a staining mixture comprises combining a buffer with a DNA selective dye to form a buffered dye solution, and combining the buffered dye solution with the sperm cells to form the staining mixture.

19. The process of claim 1, further comprising the step of combining the a quencher with the staining mixture.

20. The process of claim 19, wherein the quencher is selected from the group consisting of FD&C #40 and propidium iodide.

21. The process of claim 20, wherein the quencher is FD&C #40.

22. The process of claim 20, wherein the quencher is FD&C #40 and the dye is Hoechst 33342.

23. The process of claim 20, wherein the quencher is propidium iodide and the dye is SYBR-14.

24. The process of claim 1, wherein the staining mixture further contains a motility inhibitor.

25. The process of claim 1, wherein the step of forming a staining mixture comprises combining a motility inhibitor with the sperm cells to form an inhibited sperm suspension, and combining the inhibited sperm suspension with a DNA selective dye to form the staining mixture.



26. The process of claim 25, wherein the motility inhibitor comprises a carbonate based motility inhibitor.

27. The process of claim 26, wherein the carbonate based motility inhibitor comprises  $\text{NaHCO}_3$ ,  $\text{KHCO}_3$ , and  $\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$ .

28. The process of claim 27, wherein the carbonate based motility inhibitor comprises 0.097 moles/L of  $\text{NaHCO}_3$ , 0.173 moles/L of  $\text{KHCO}_3$ , 0.090 moles/L  $\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$  in water.

29. The process of claim 1, wherein the staining mixture further contains a composition which regulates oxidation/reduction reactions intracellularly or extracellularly.

30. The process of claim 29, wherein the composition which regulates oxidation/reduction reactions intracellularly or extracellularly is selected from the group consisting of pyruvate, vitamin K, lipoic acid, glutathione, flavins,  
5 quinones, superoxide dismutase, and superoxide dismutase mimics.

31. The process of claim 30, wherein the composition which regulates oxidation/reduction reactions intracellularly or extracellularly is selected from the group consisting of pyruvate, vitamin K, and lipoic acid.

32. The process of claim 31, wherein the composition which regulates oxidation/reduction reactions intracellularly or extracellularly comprises pyruvate at a concentration from about  $0.5\mu\text{M}$  to about 50mM.

33. The process of claim 32, wherein the composition which regulates oxidation/reduction reactions intracellularly or

extracellularly comprises pyruvate at a concentration from about 10mM to about 15mM.

34. The process of claim 33, wherein the composition which regulates oxidation/reduction reactions intracellularly or extracellularly comprises pyruvate at a concentration of about 10mM.

35. The process of claim 31, wherein the composition which regulates oxidation/reduction reactions intracellularly or extracellularly comprises vitamin K at a concentration of about 1 $\mu$ M to about 100 $\mu$ M.

36. The process of claim 35, wherein the composition which regulates oxidation/reduction reactions intracellularly or extracellularly comprises vitamin K at a concentration of about 100 $\mu$ M.

37. The process of claim 31, wherein the composition which regulates oxidation/reduction reactions intracellularly or extracellularly comprises lipoic acid at a concentration of about 0.1mM to about 1.0mM.

38. The process of claim 31, wherein the composition which regulates oxidation/reduction reactions intracellularly or extracellularly comprises lipoic acid at a concentration of about 1.0mM.

FIG. 1A

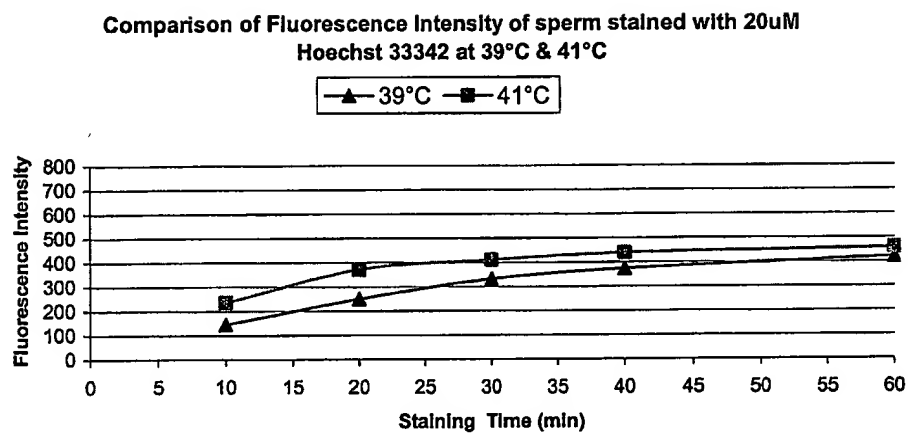
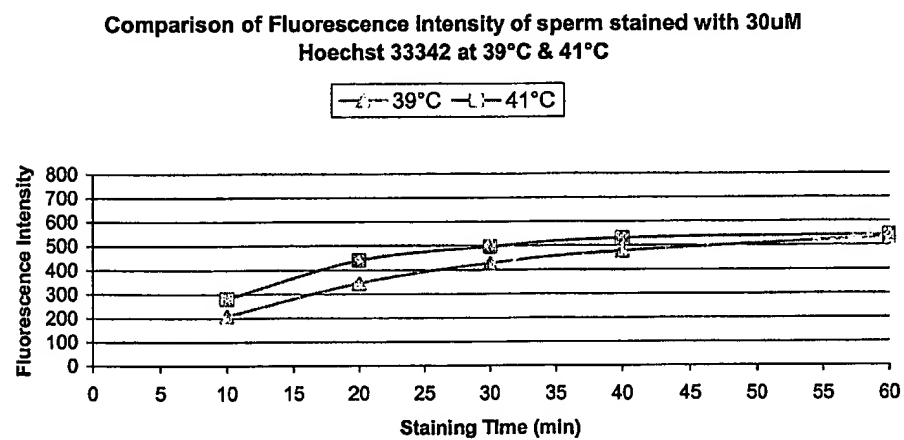
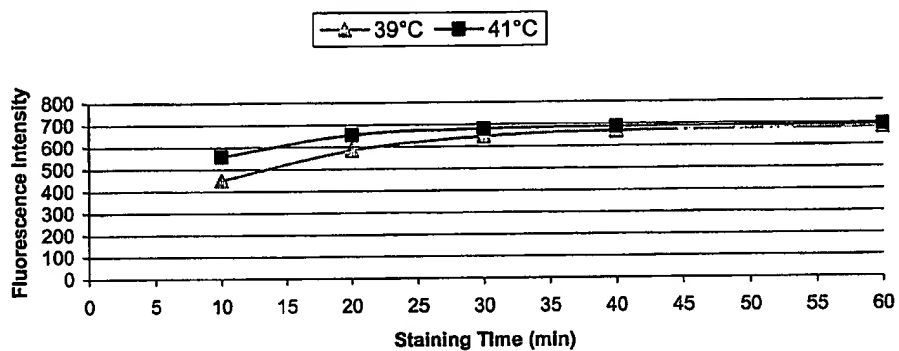


FIG. 1B



## FIG. 1C

Comparison of Fluorescence Intensity of sperm stained with 57uM Hoechst 33342 at 39°C & 41°C



## FIG. 1D

Comparison of Fluorescence Intensity of sperm stained with 85uM Hoechst 33342 at 39°C & 41°C

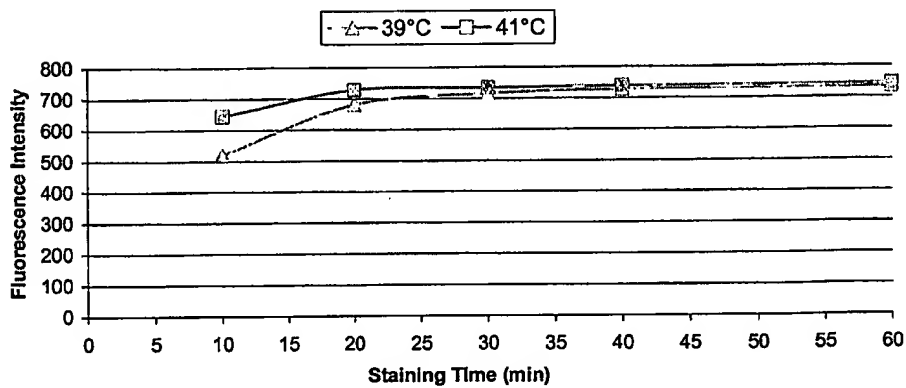


FIG. 2A

Comparison of Fluorescence Intensity of sperm stained with 20uM Hoechst 33342 at 39°C & 41°C

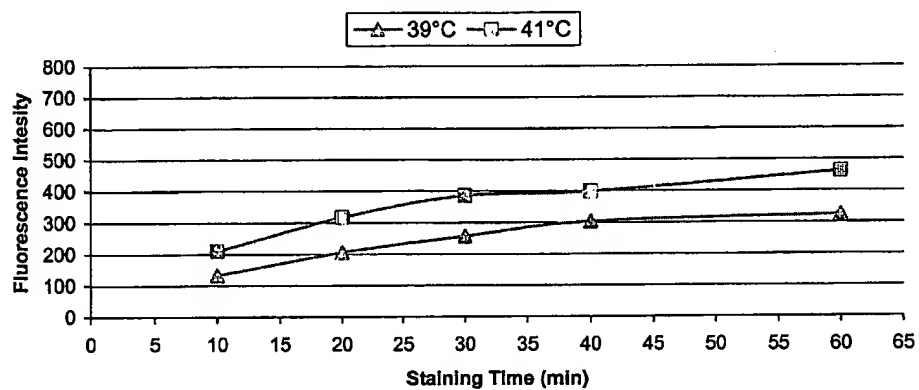


FIG. 2B

Comparison of Fluorescence Intensity of sperm stained with 30uM Hoechst 33342 at 39°C & 41°C

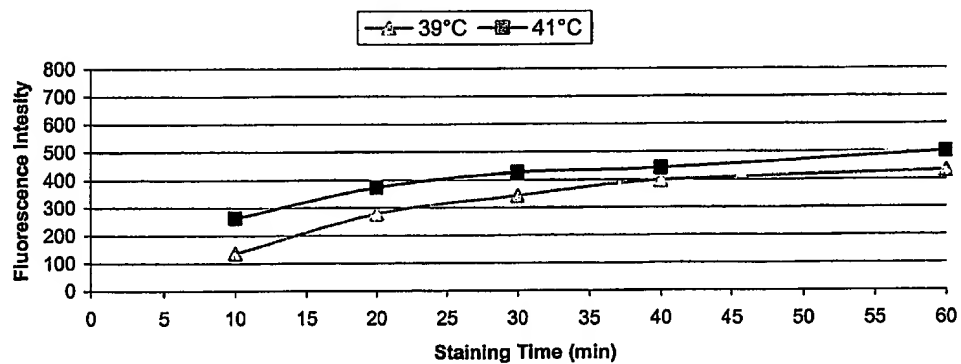


FIG. 2C

Comparison of Fluorescence Intensity of sperm stained with 40uM Hoechst 33342 at 39°C & 41°C

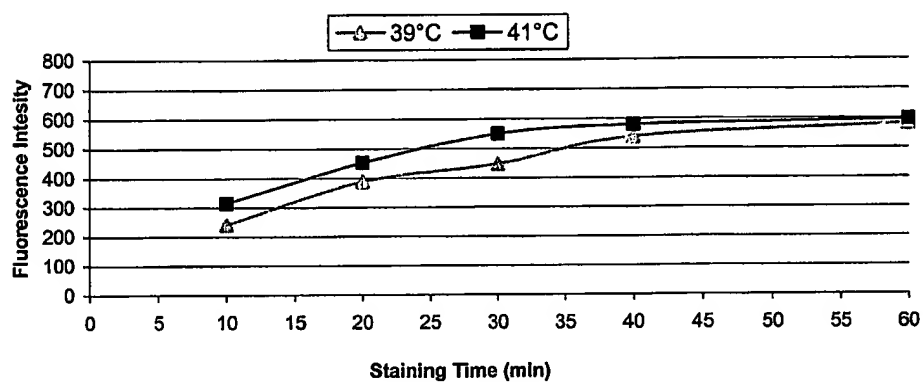


FIG. 2D

Comparison of Fluorescence Intensity of sperm stained with 60uM Hoechst 33342 at 39°C & 41°C

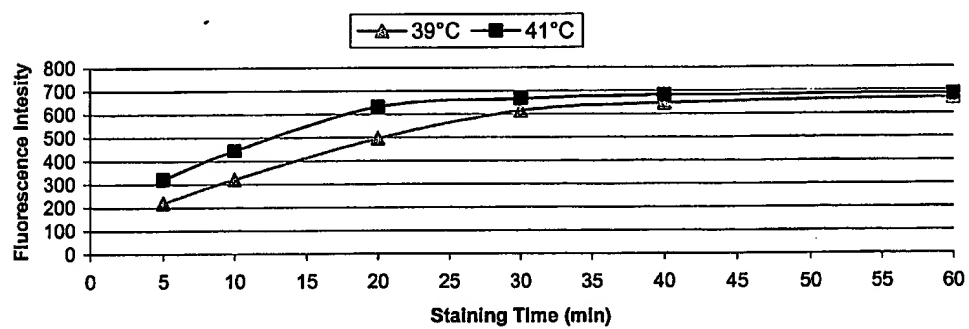


FIG. 3A

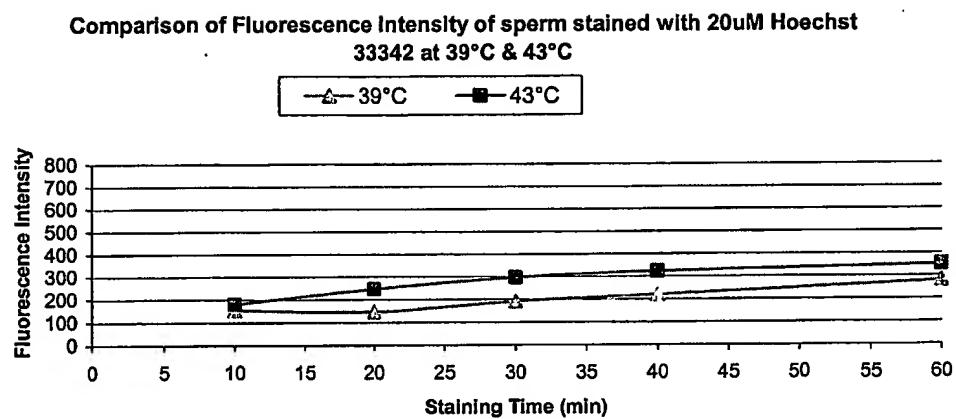


FIG. 3B

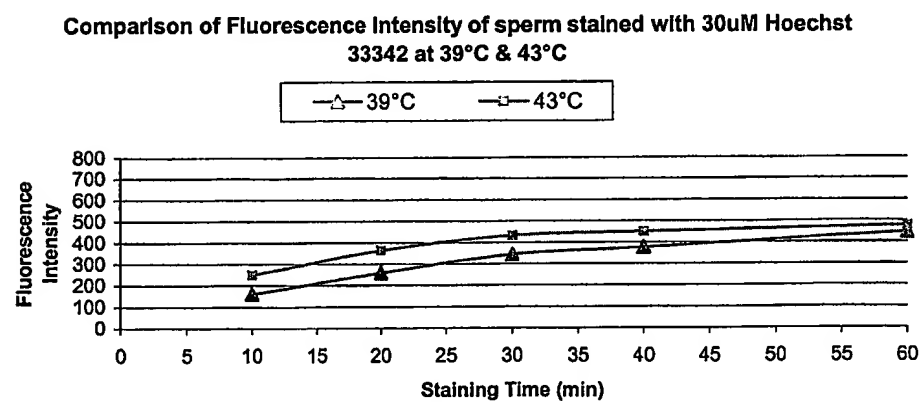


FIG. 3C

Comparison of Fluorescence Intensity of sperm stained with 40uM  
Hoechst 33342 at 39°C & 43°C

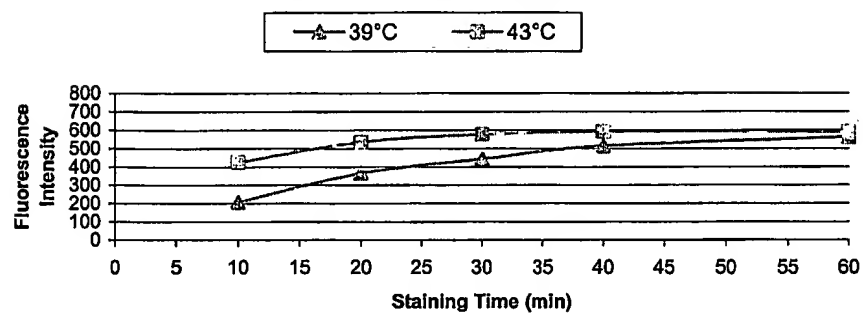


FIG. 3D

Comparison of Fluorescence Intensity of sperm stained with 60uM  
Hoechst 33342 at 39°C & 43°C

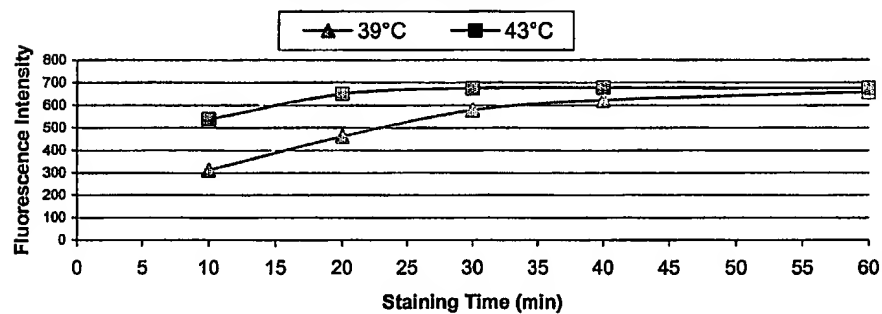




FIG. 4A

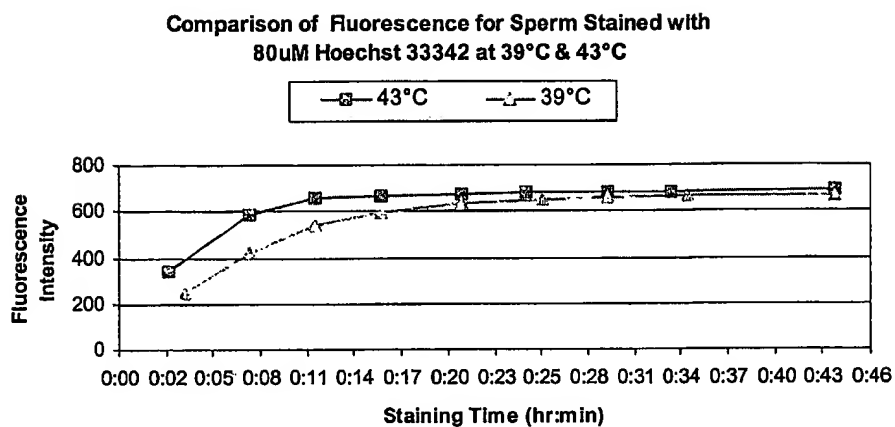


FIG. 4B

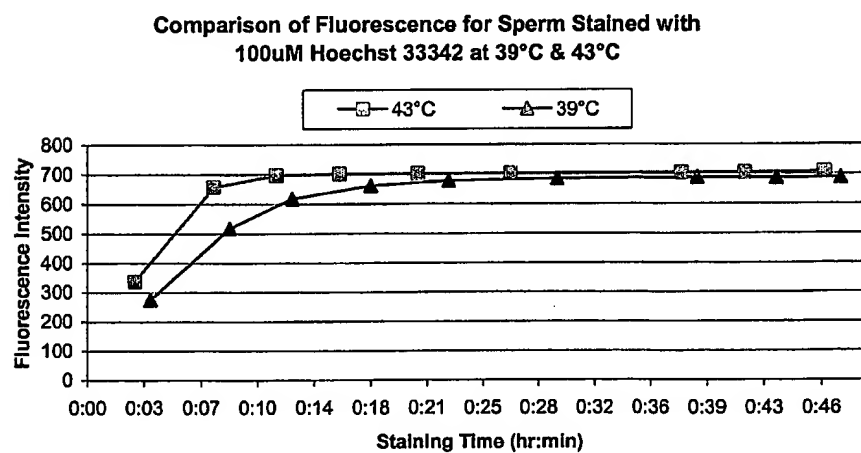


FIG. 5A

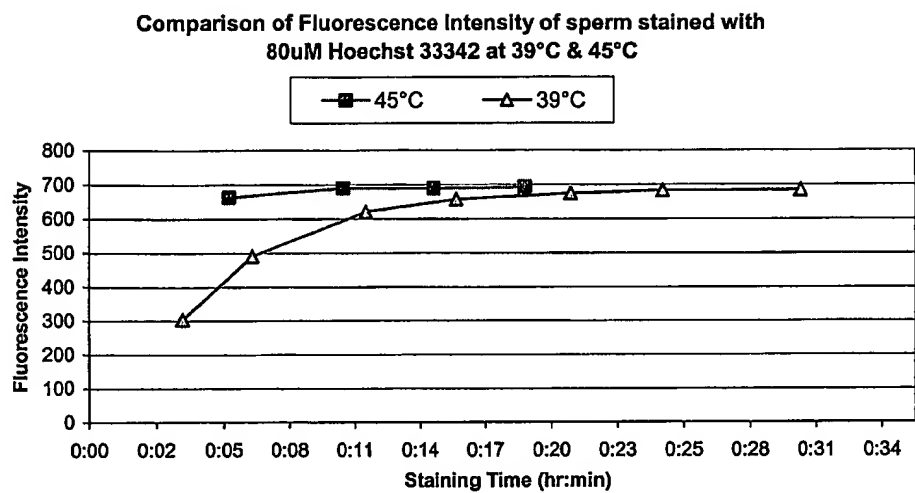


FIG. 5B

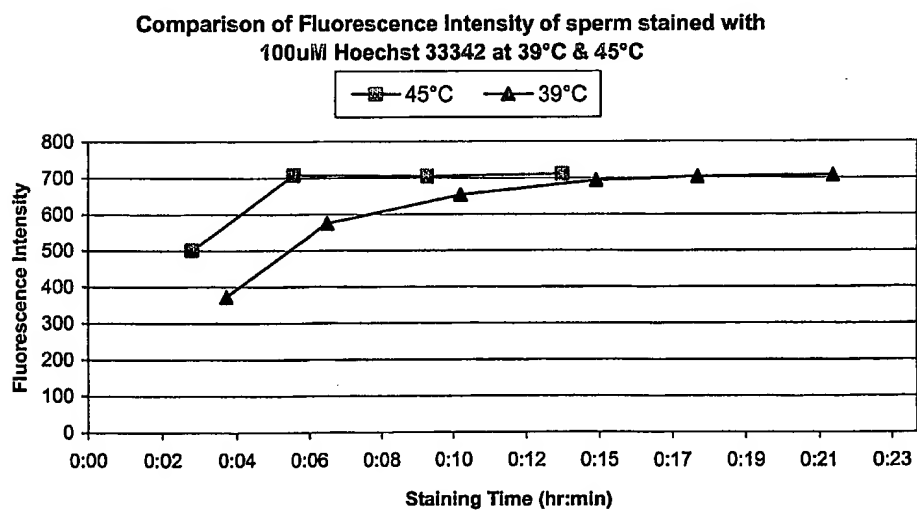


FIG. 6A

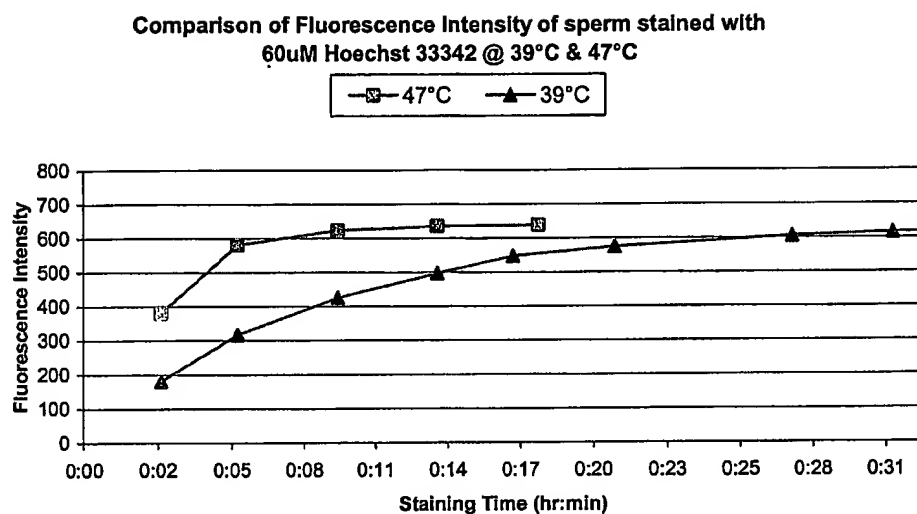


FIG. 6B

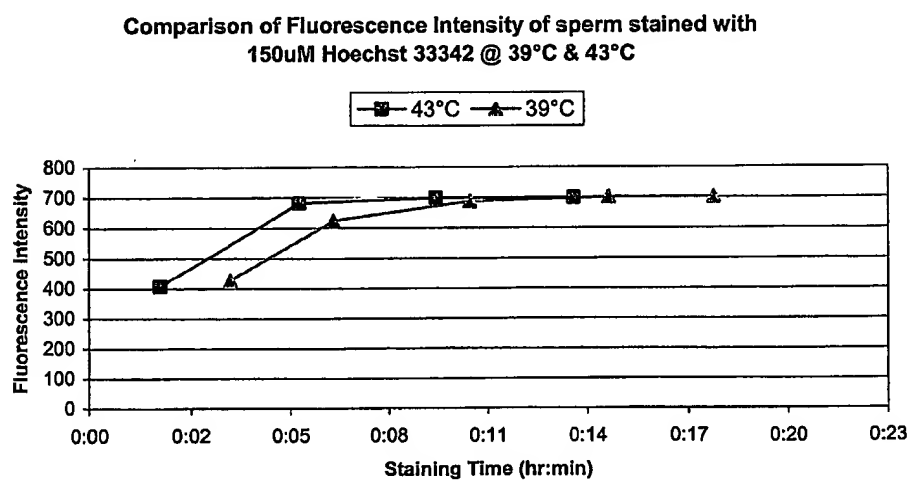


FIG. 7A

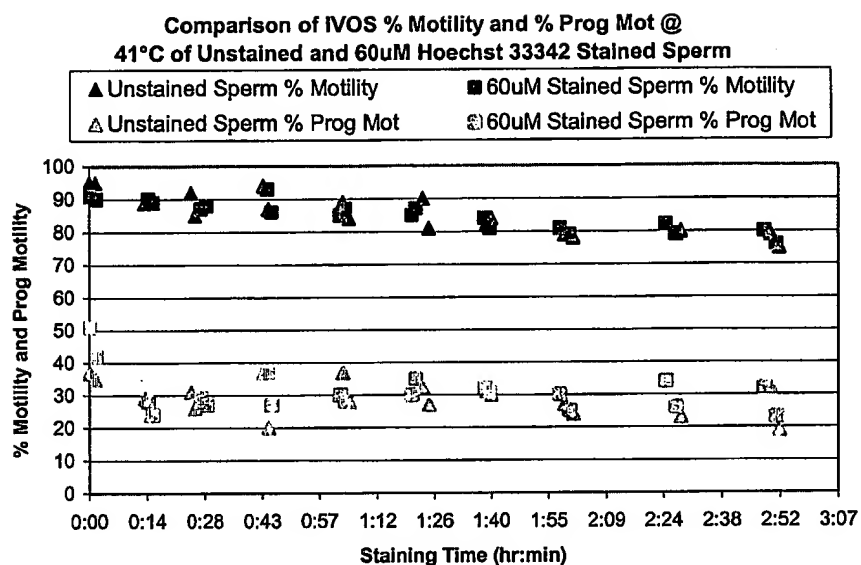


FIG. 7B

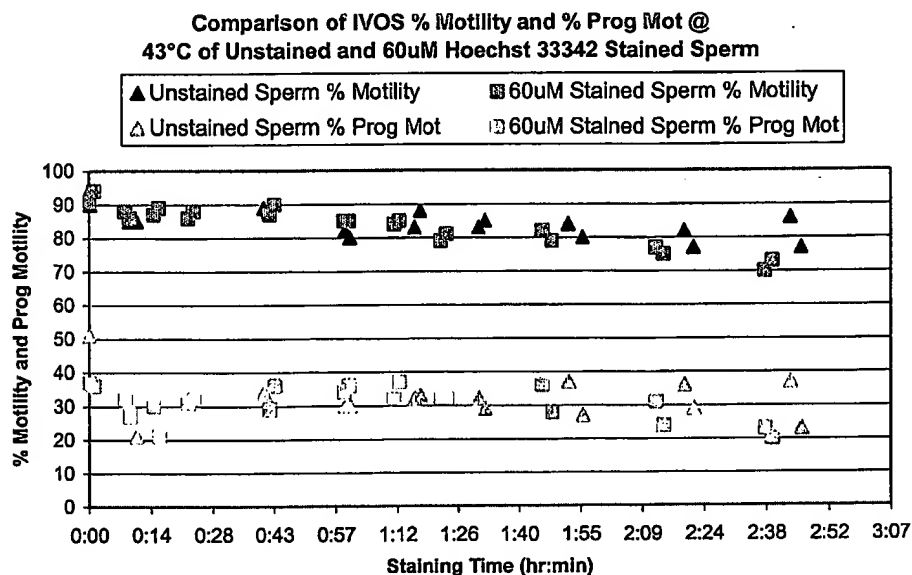


FIG. 8A

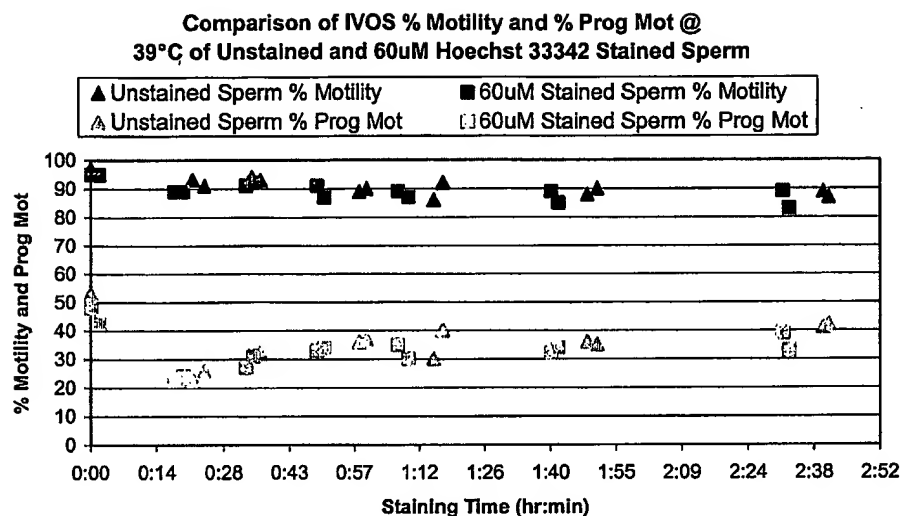


FIG. 8B

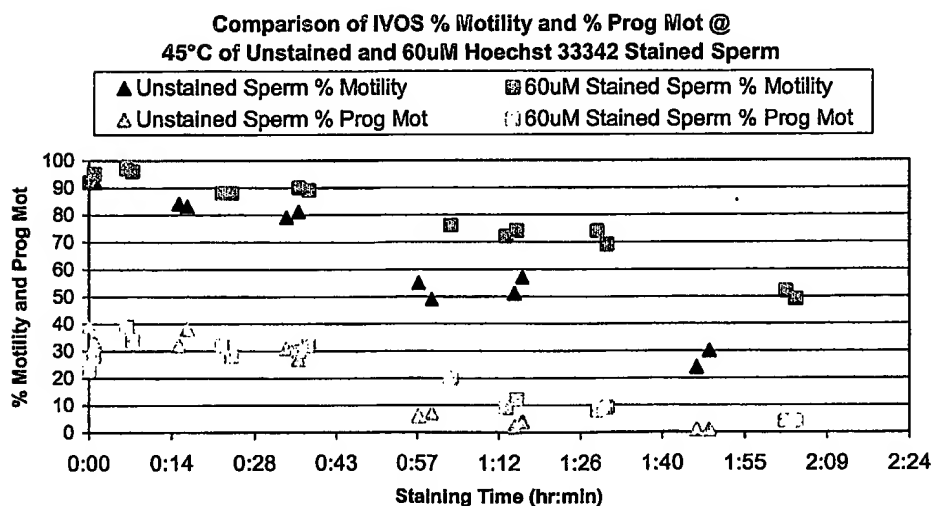


FIG. 9A

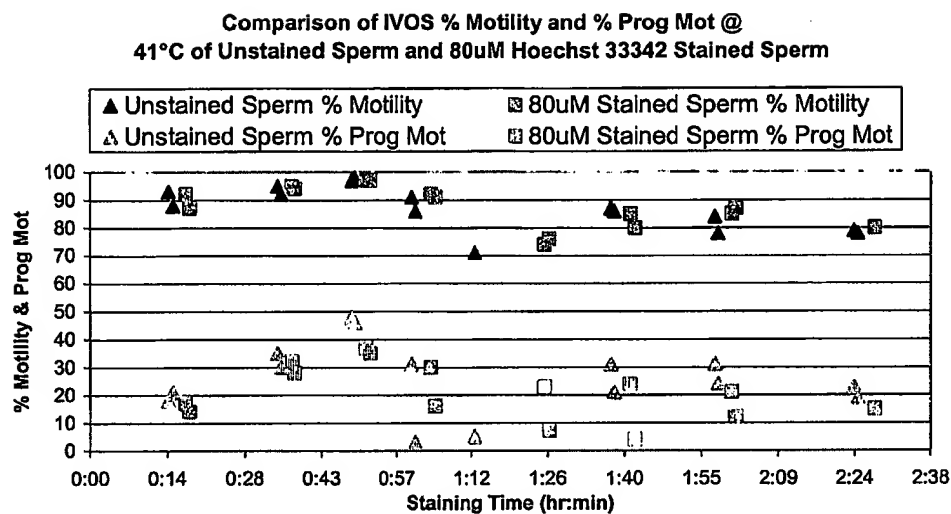


FIG. 9B

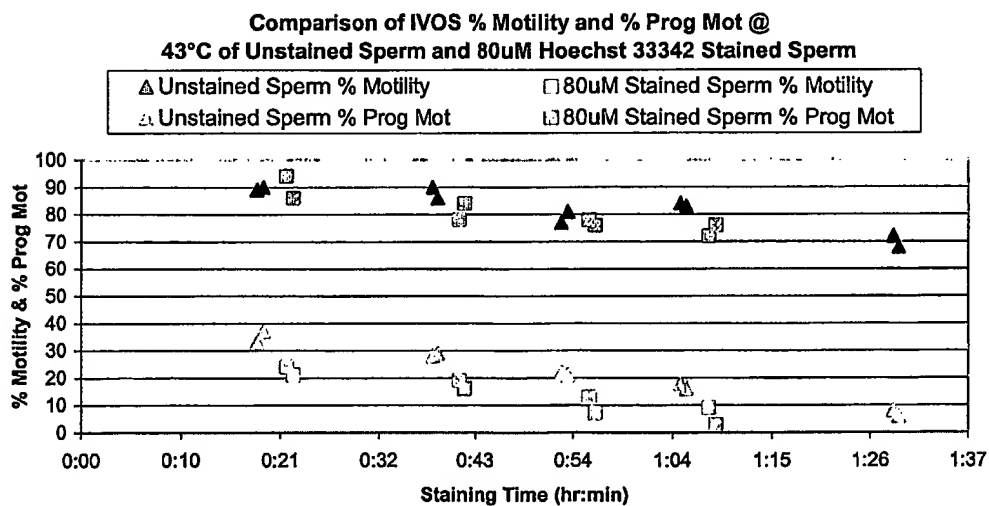


FIG. 9C

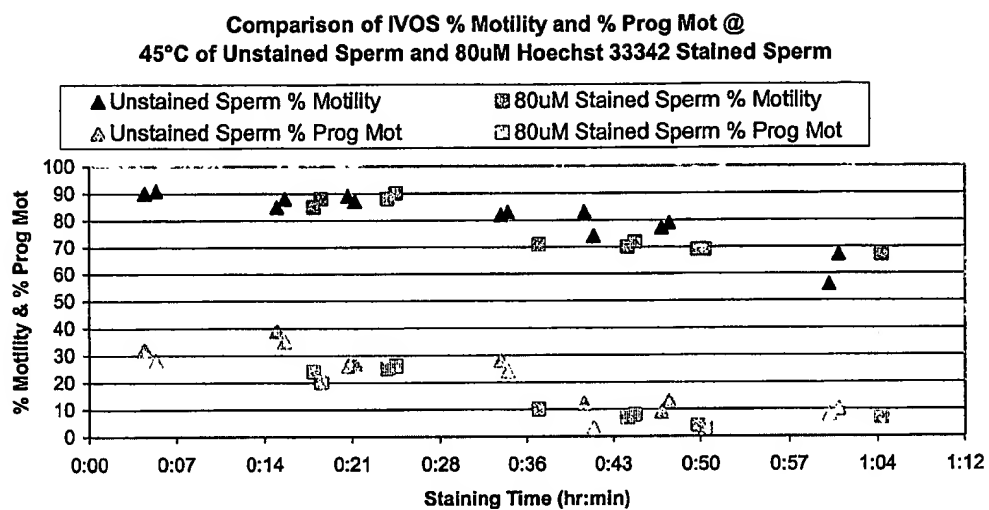


FIG. 10A

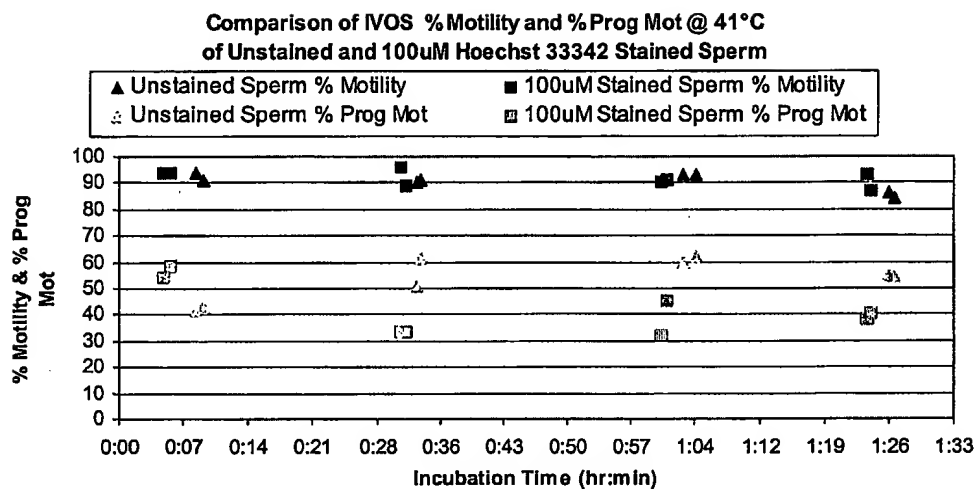


FIG. 10B

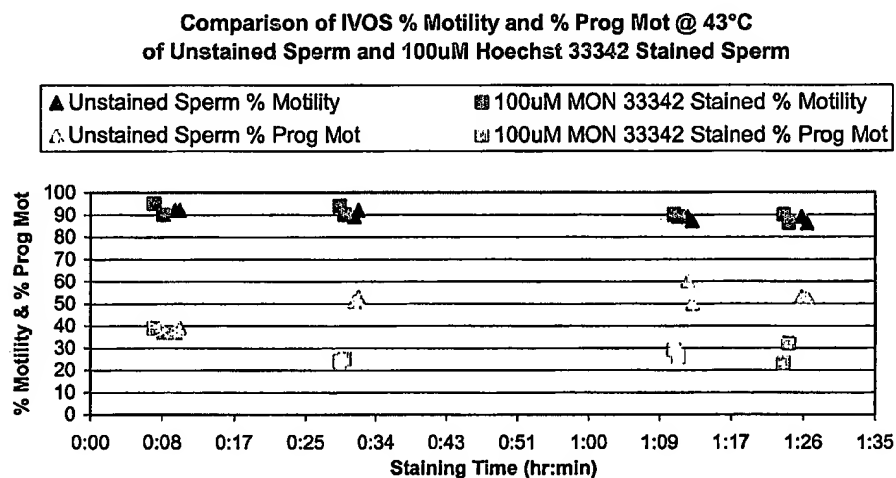




FIG. 10C

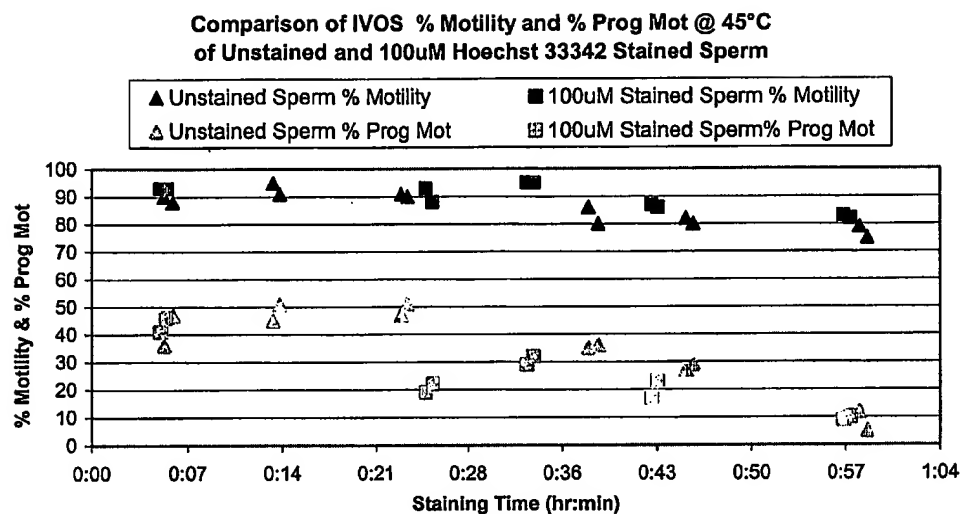


FIG. 11

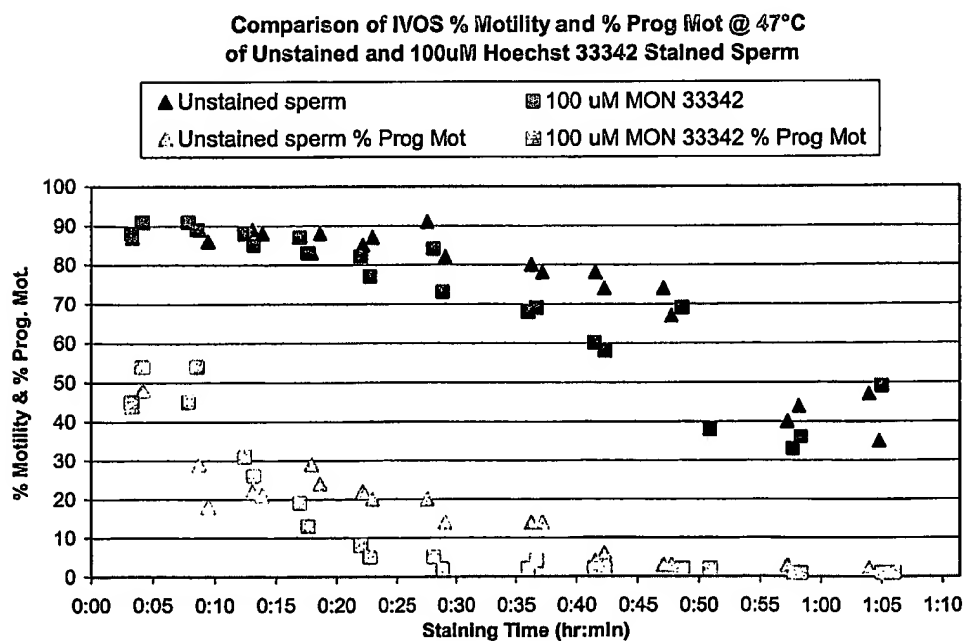


FIG. 12

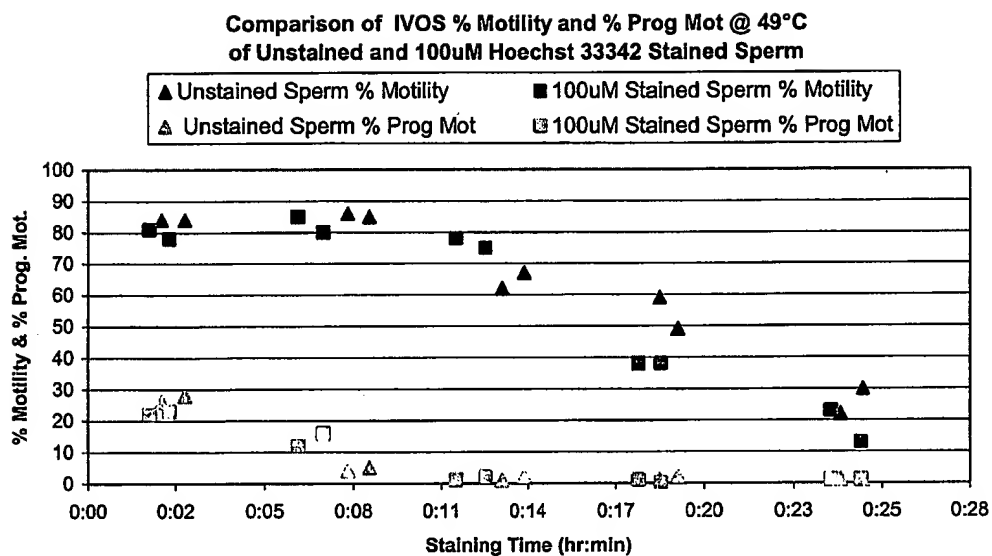


FIG. 13A

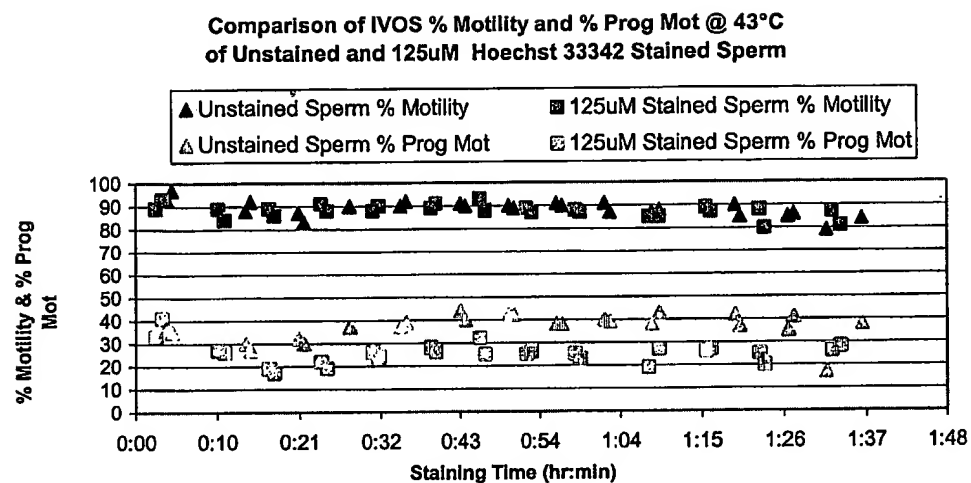


FIG. 13B

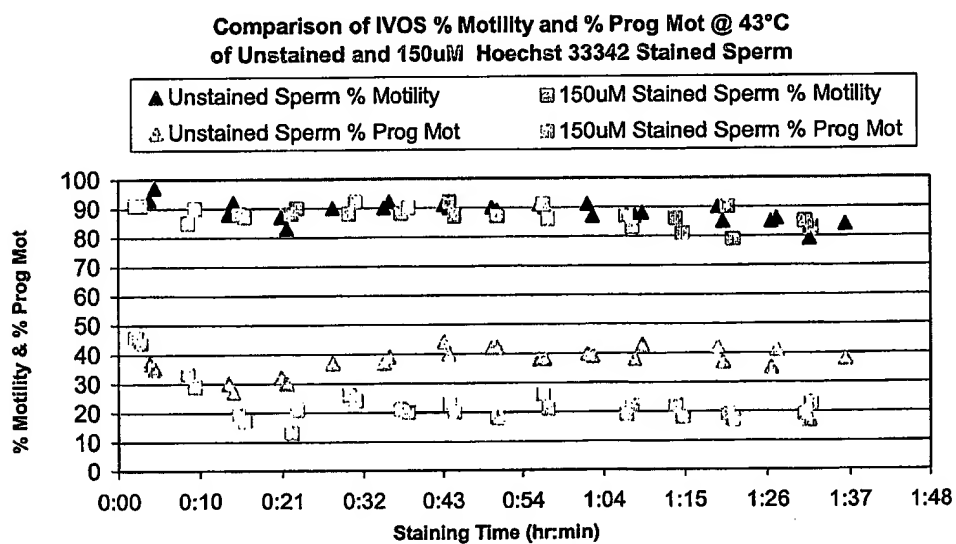


FIG. 14A

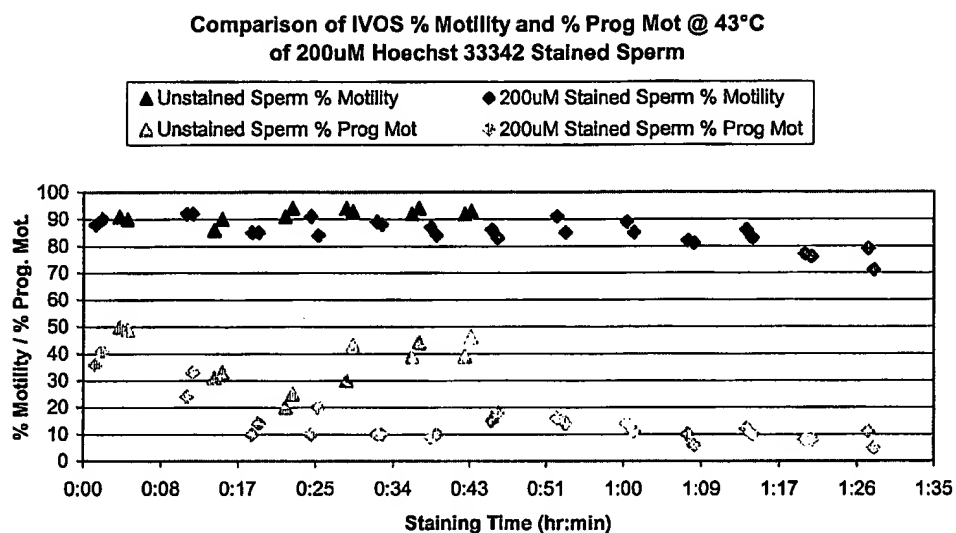


FIG. 14B

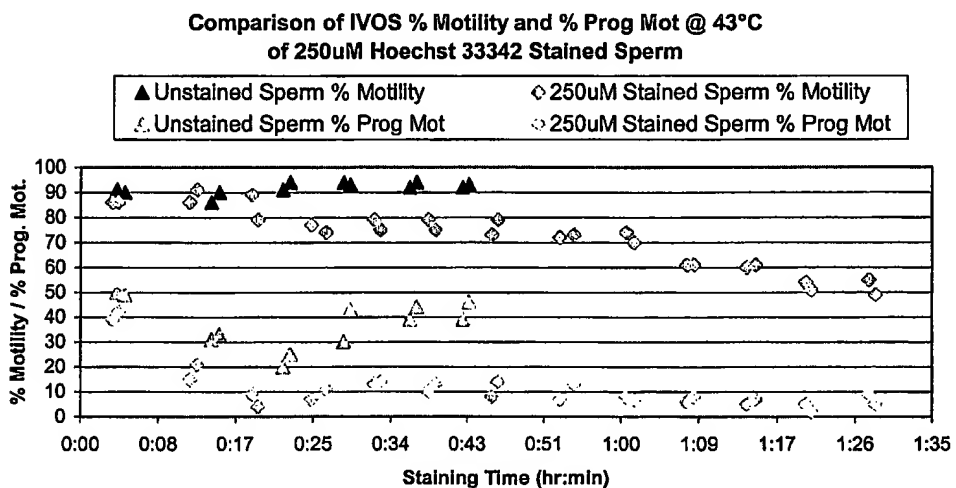


FIG. 15A

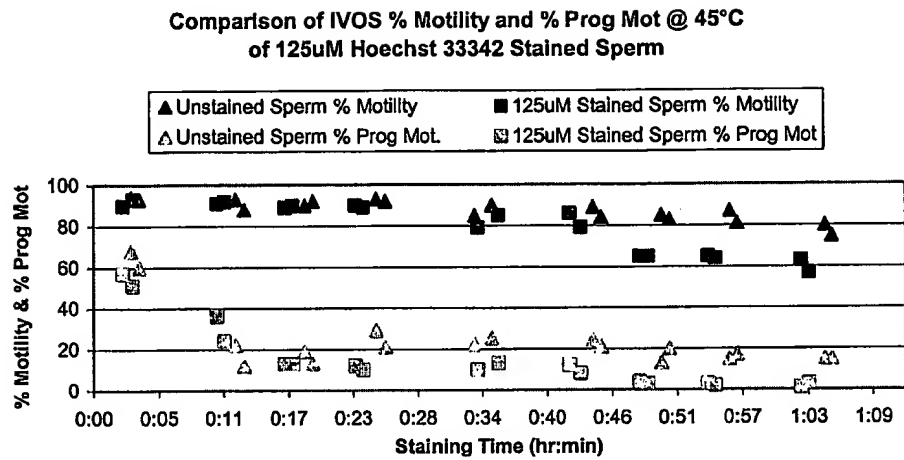


FIG. 15B

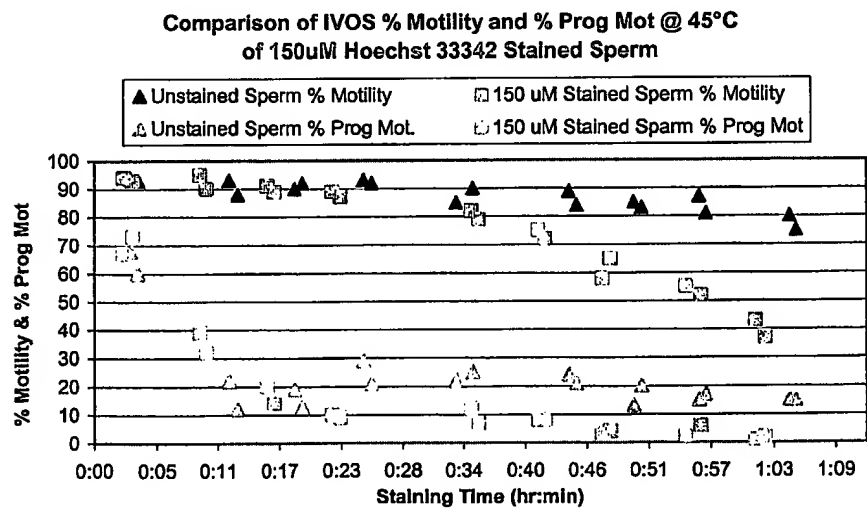


FIG. 16A

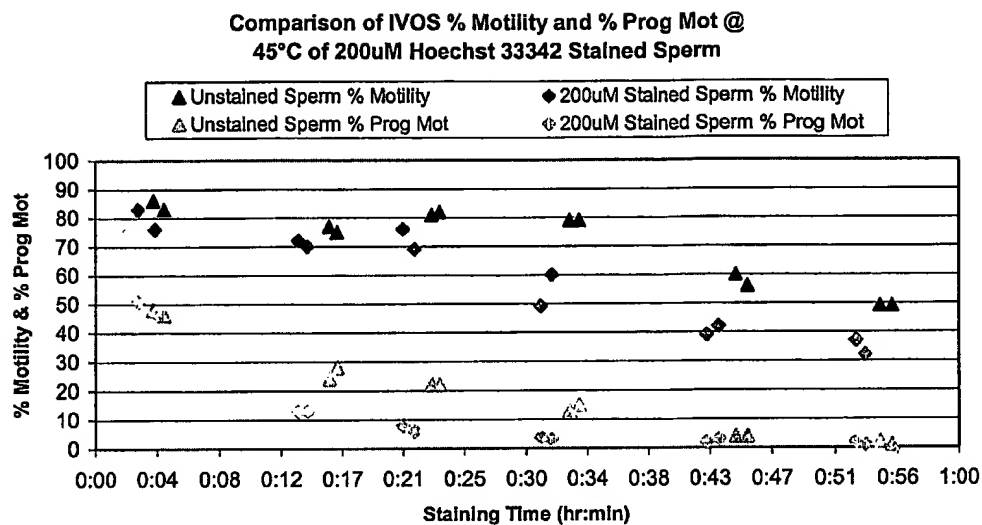


FIG. 16B

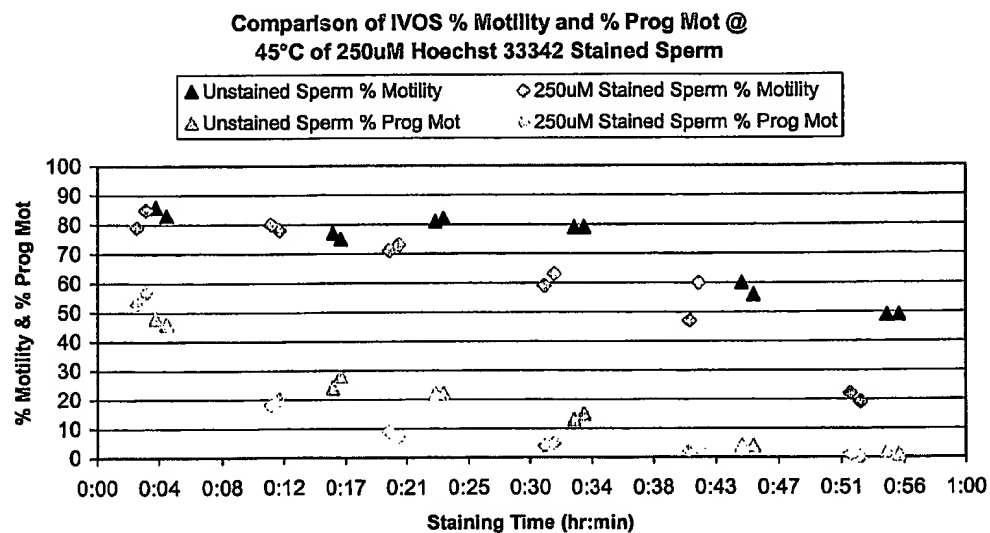


FIG. 16C

Comparison of IVOS % Motility and % Prog Mot @  
45°C of 300uM Hoechst 33342 Stained Sperm

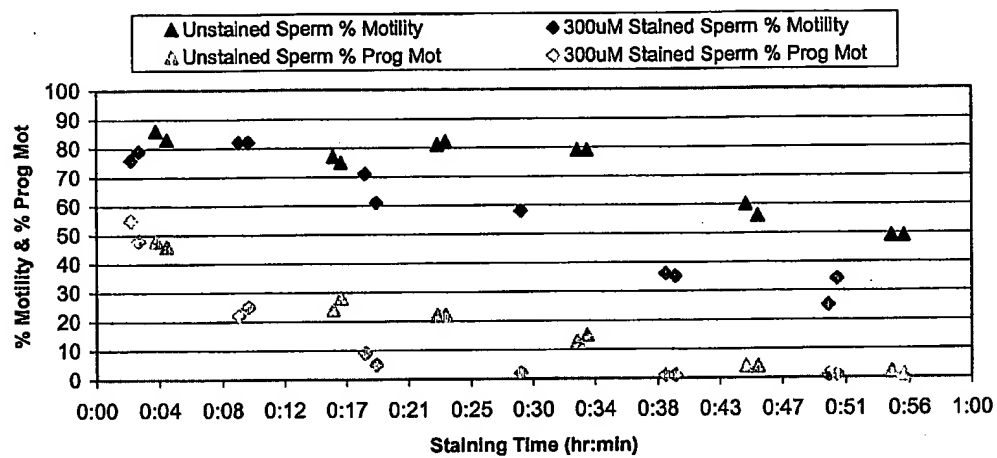


FIG. 17A

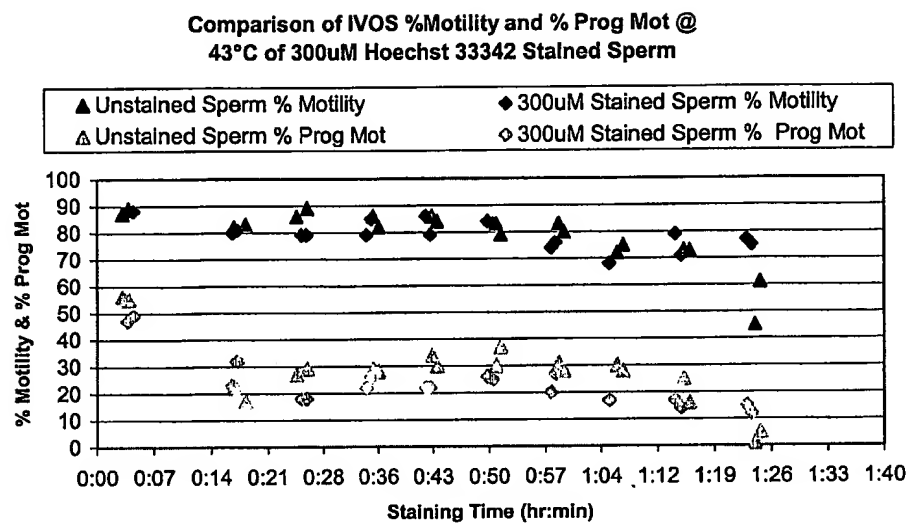


FIG. 17B

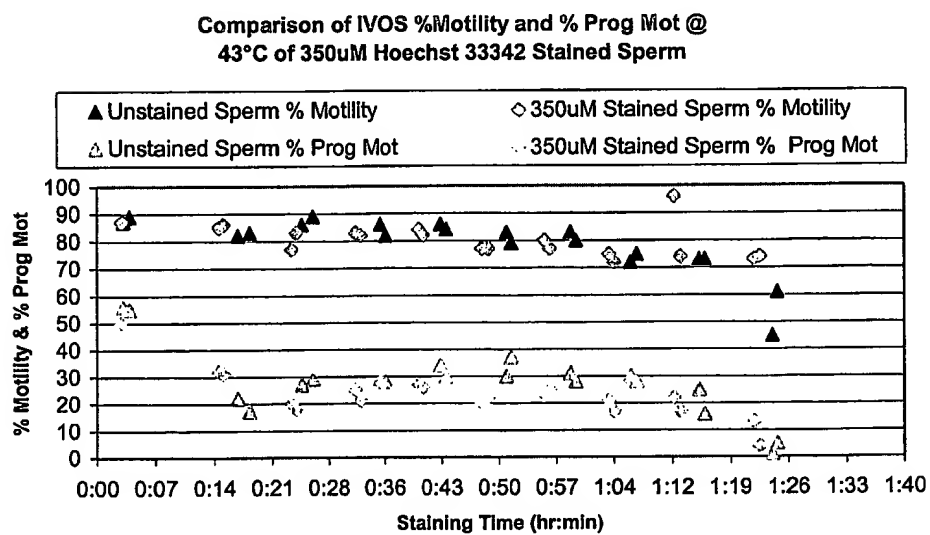




FIG. 17C

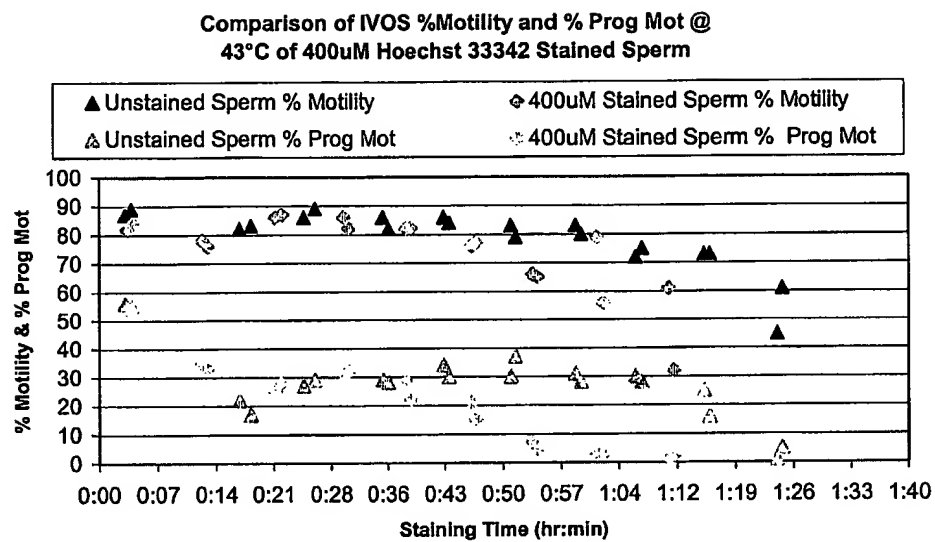


FIG. 18A

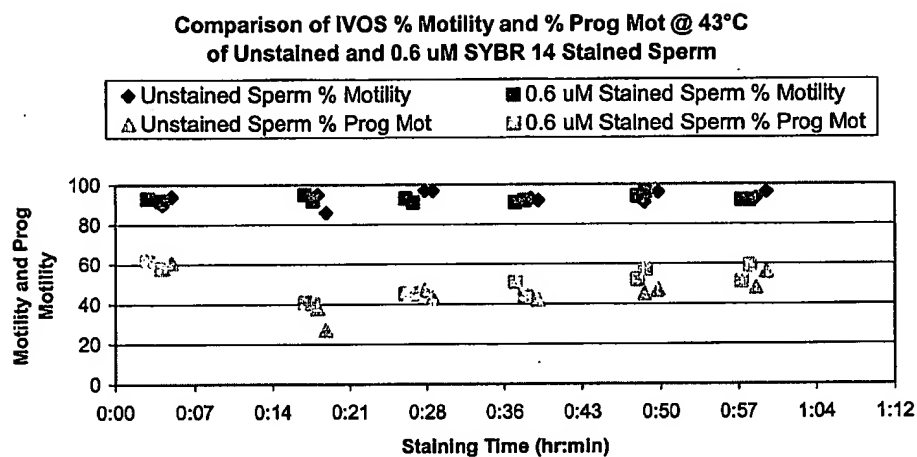


FIG. 18B

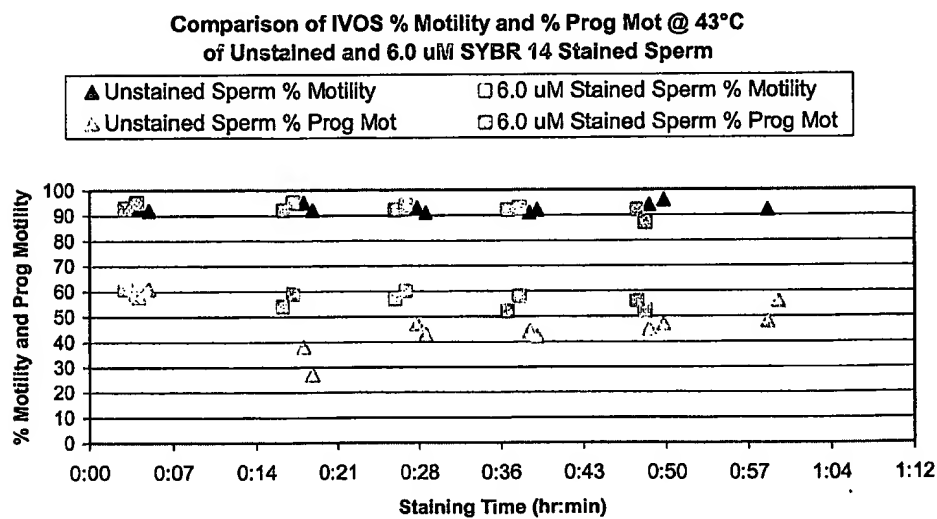


FIG. 18C

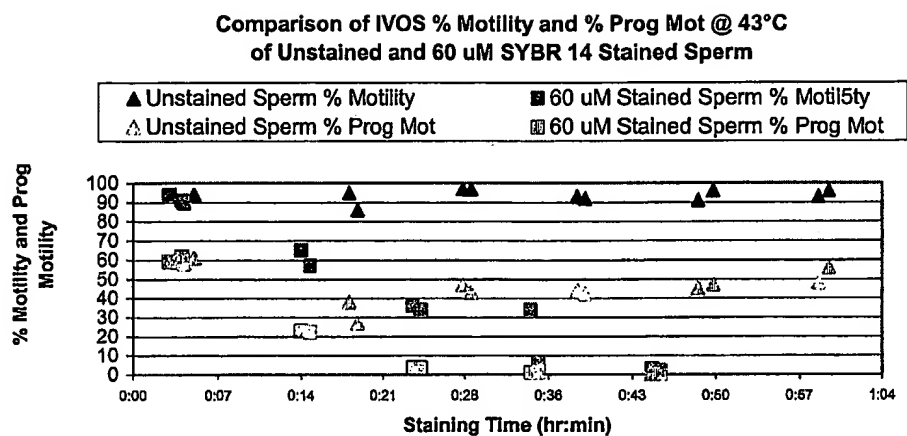


FIG. 19A

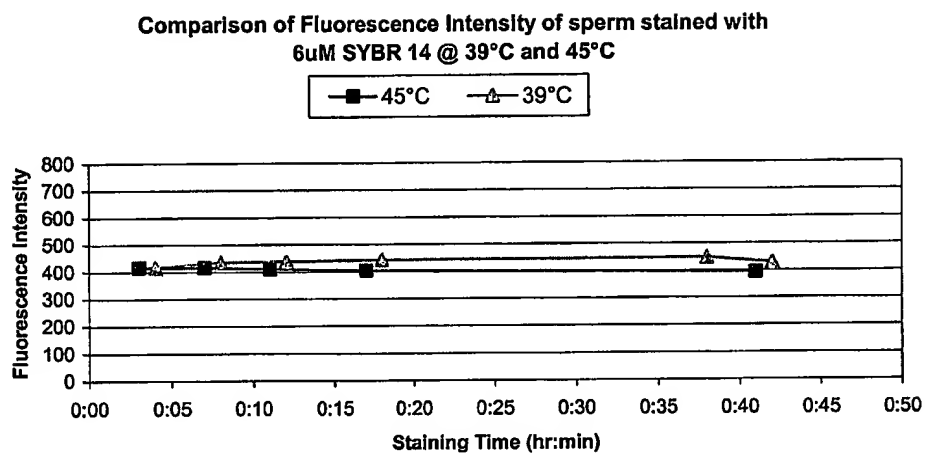


FIG. 19B

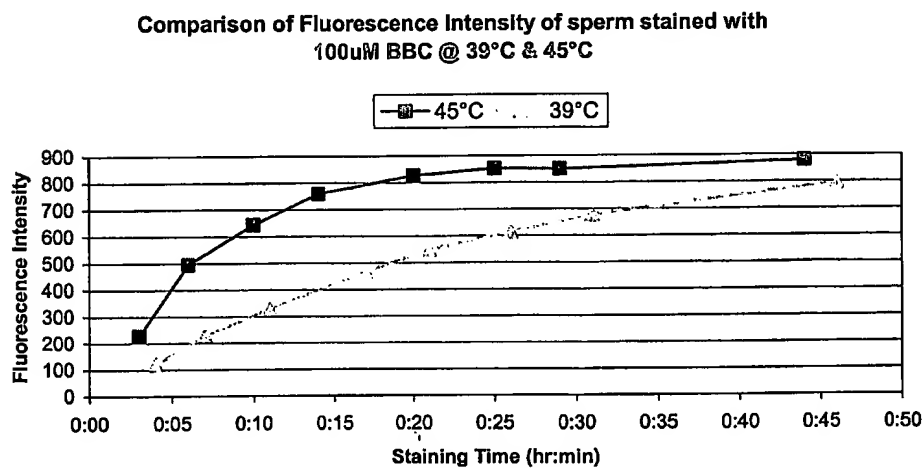


FIG. 20

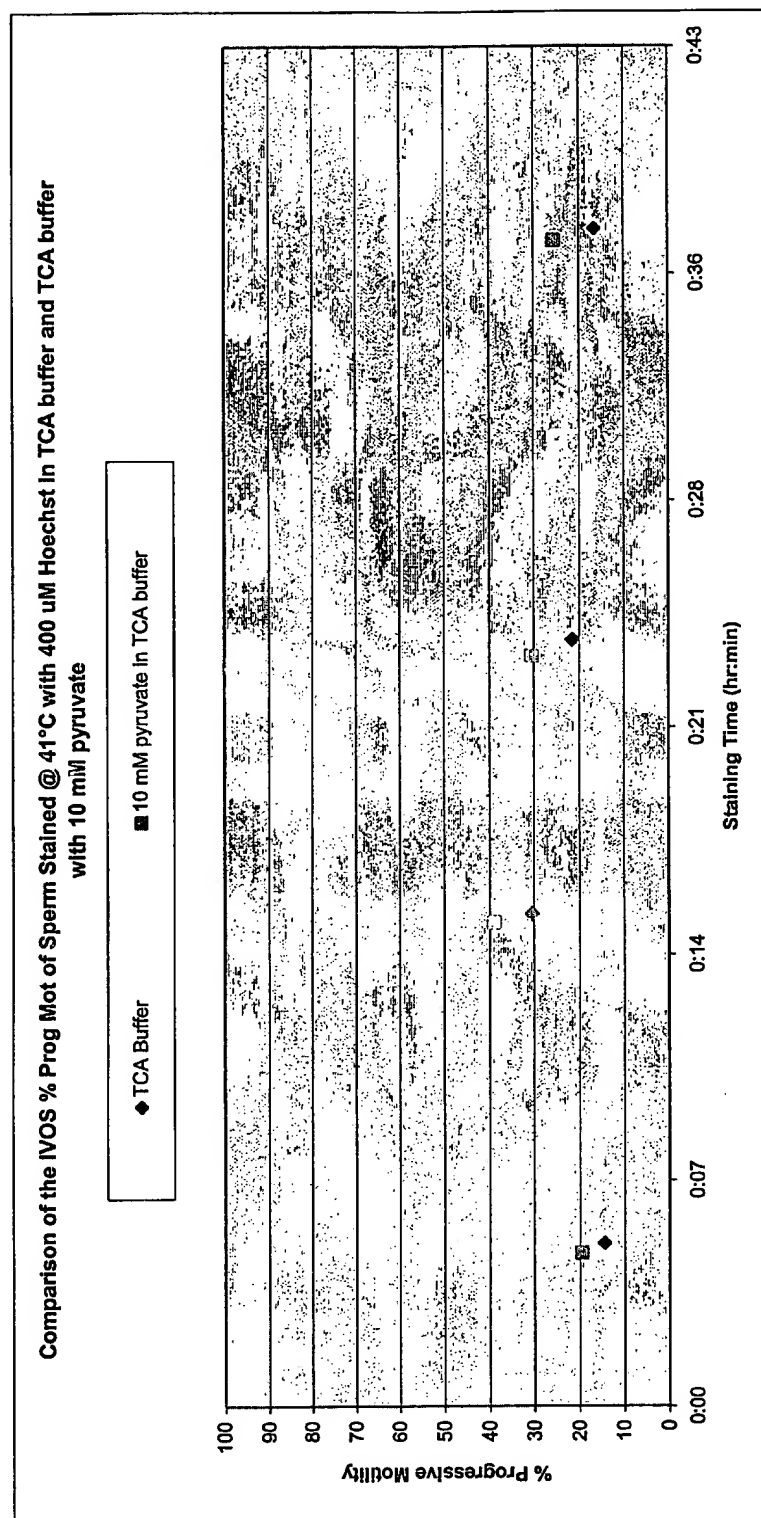


FIG. 21

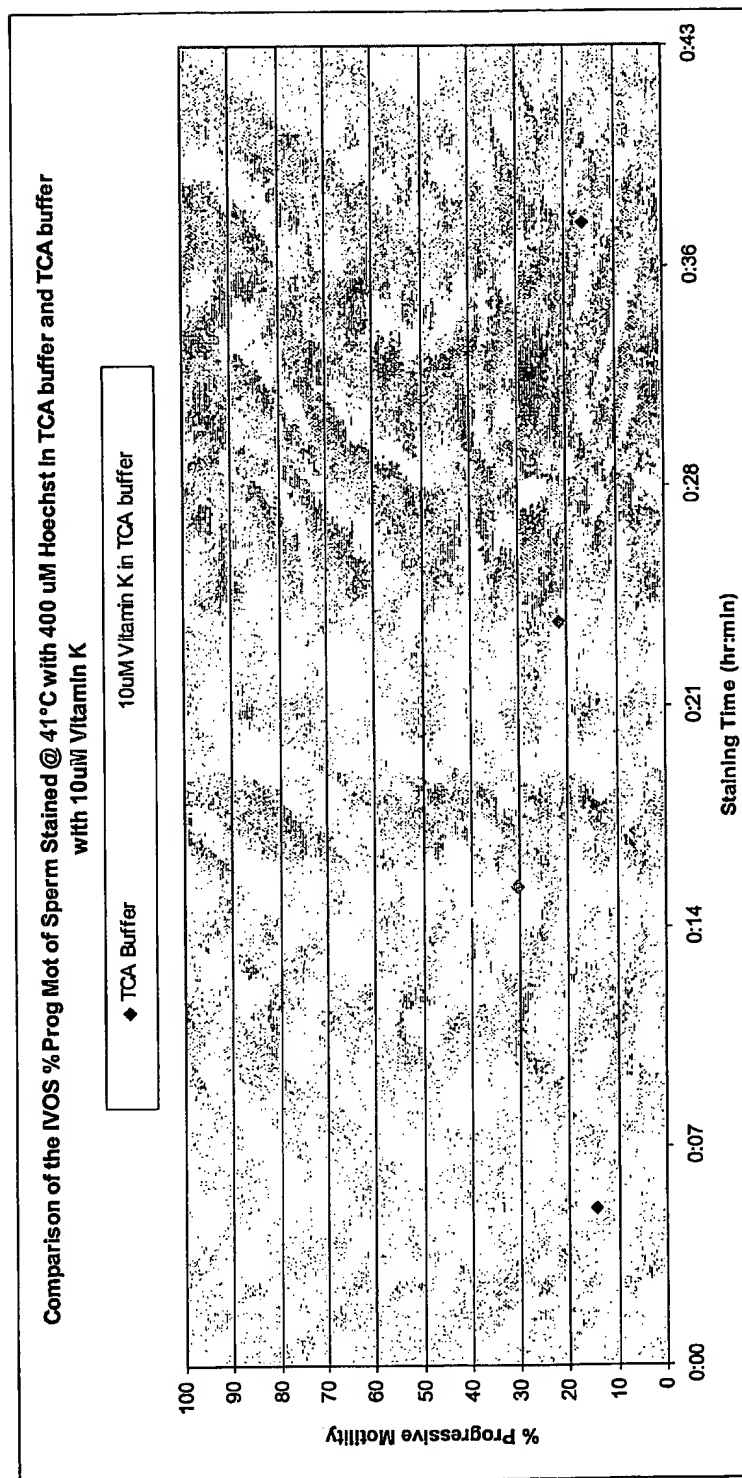


FIG. 22

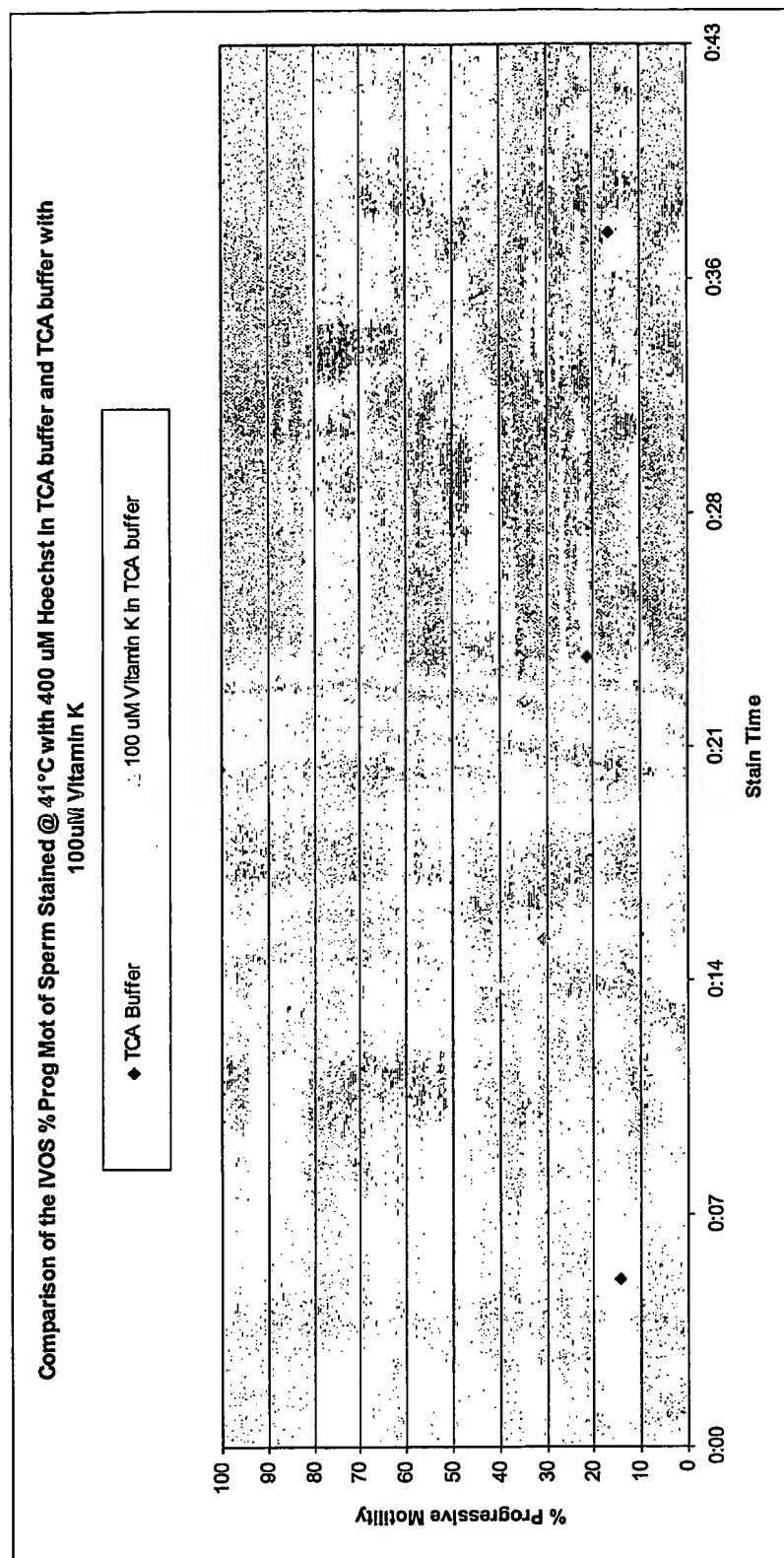


FIG. 23

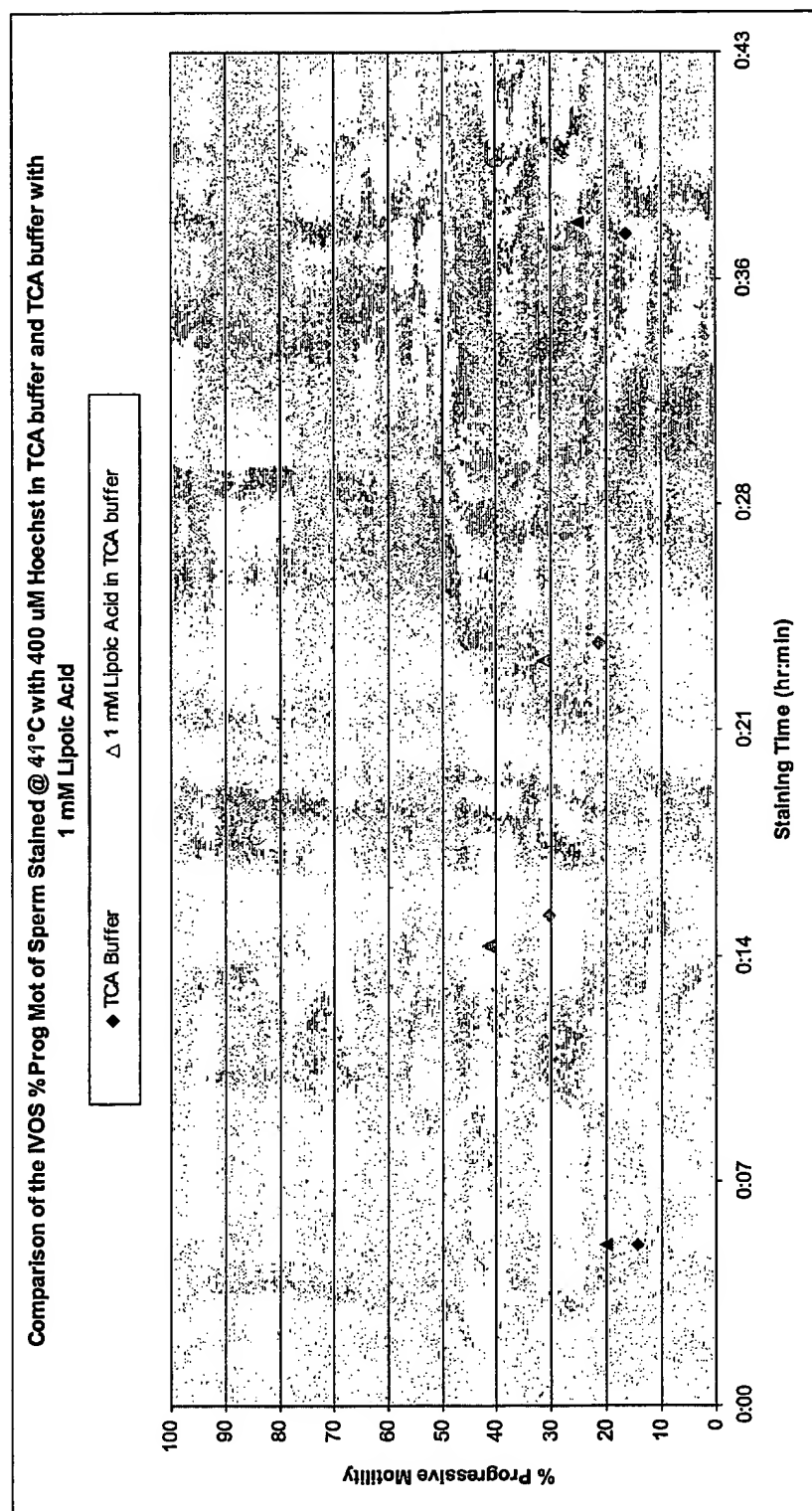




FIG. 24

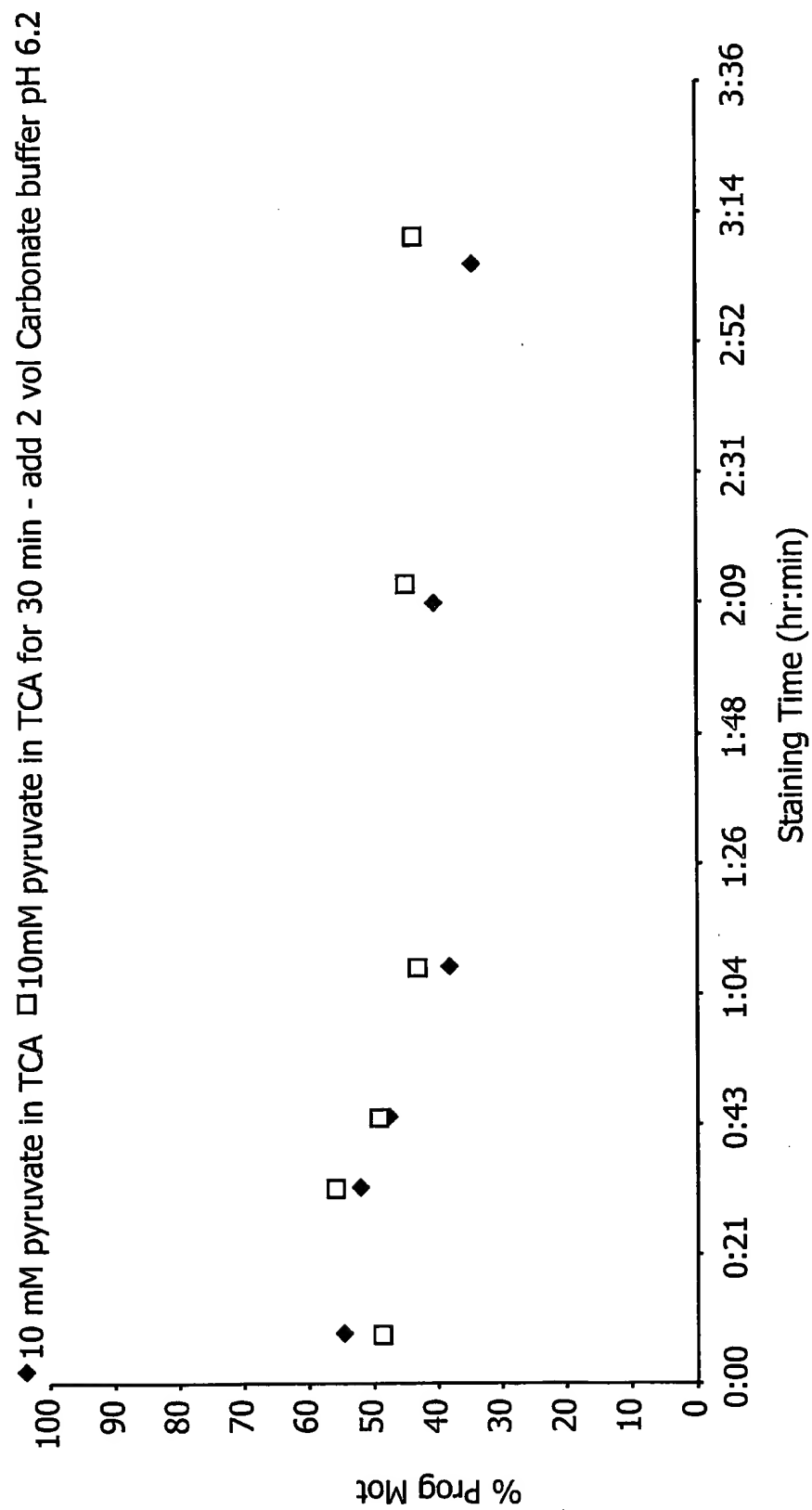


FIG. 25

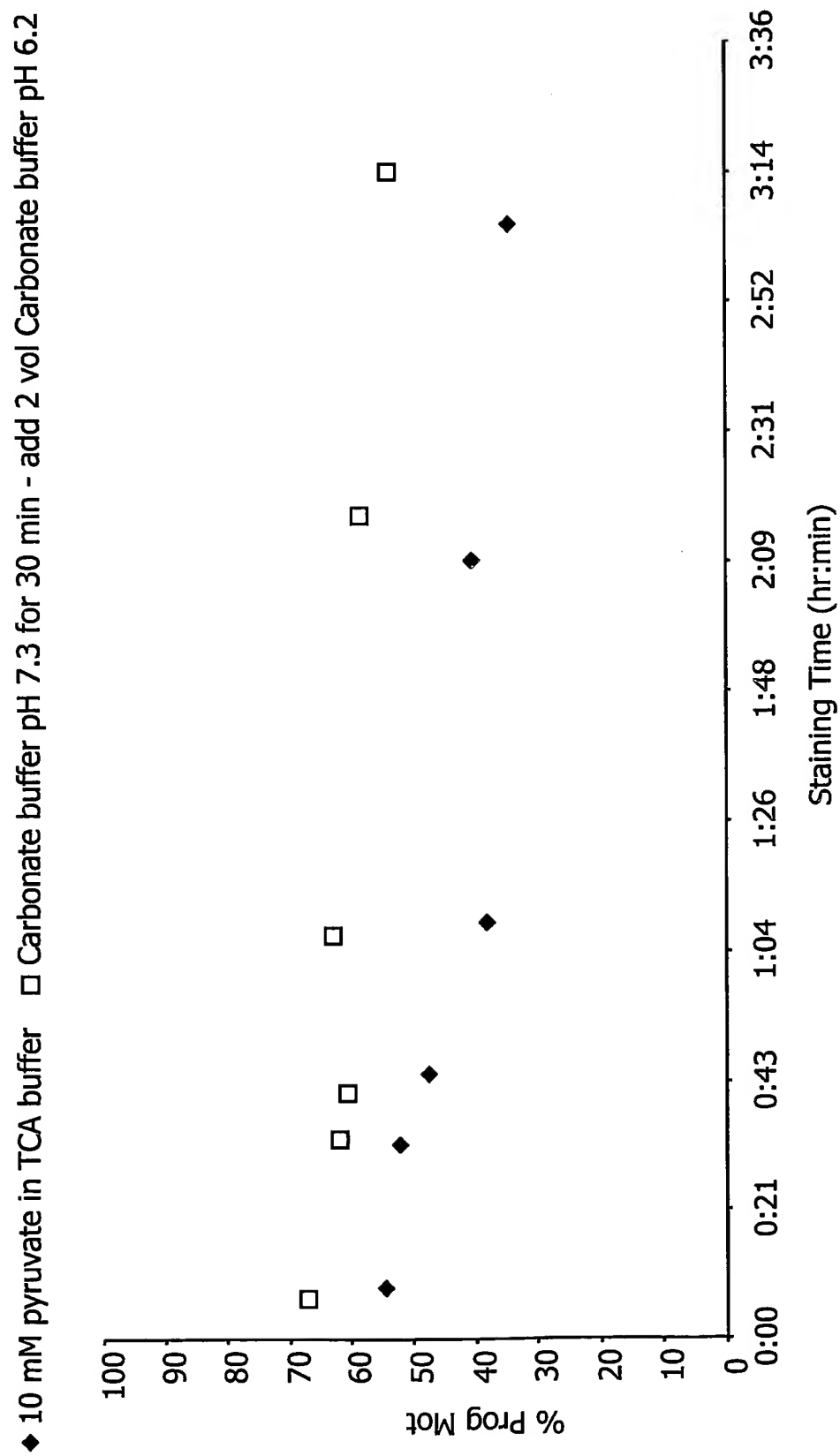
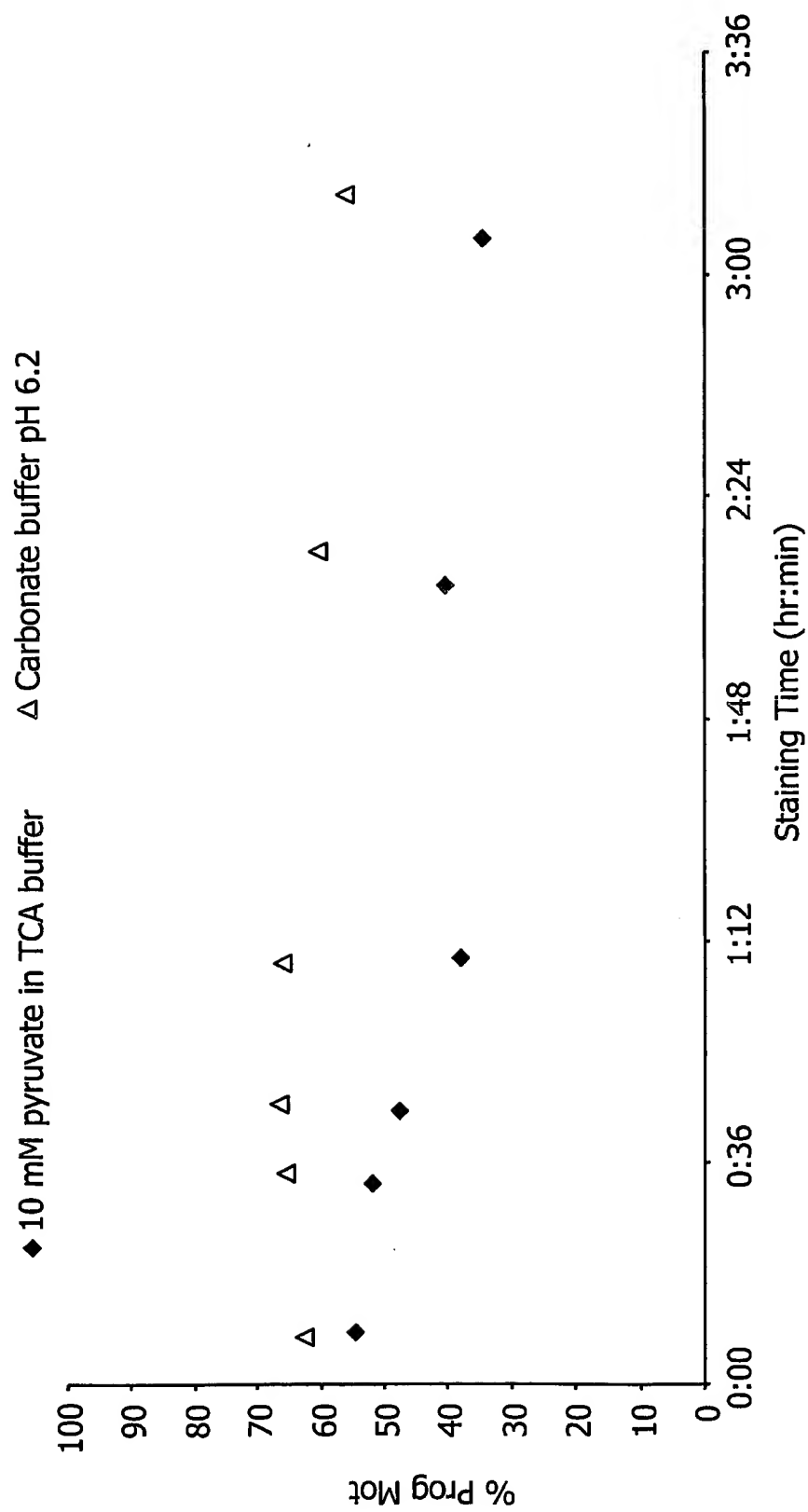


FIG. 26



# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US2004/009903

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K35/52 A61D19/00 C12N5/06

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K A61D C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No. |
|------------|---|-----------------------|
| A          | JOHNSON L A: "SEX PRESELECTION BY FLOW CYTOMETRIC SEPARATION OF X AND Y CHROMOSOME-BEARING SPERM BASED ON DNA DIFFERENCE: A REVIEW" REPRODUCTION, FERTILITY AND DEVELOPMENT, CSIRO, EAST MELBOURNE, AU, vol. 7, no. 4, 1995, pages 893-903, XP002915903<br>ISSN: 1031-3613<br>page 900, column 1, line 14 - line 50 | 1-38                  |
| A          | WO 01/51612 A (ALEANDRI RICCARDO ; GALLI ANDREA (IT); ISTITUTO SPERIMENTALE ITALIANO) 19 July 2001 (2001-07-19) p. 4, lines 6 - 32, p. 6, line 5 - p. 8, line 16, example 2, claims 6 - 8<br>-----<br>-/--  | 1-38                  |

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*G\* document member of the same patent family

Date of the actual completion of the international search

30 July 2004

Date of mailing of the international search report

16/08/2004

Name and mailing address of the ISA

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Fax: (+31-70) 340-3016

Authorized officer

Hoese1, H

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US2004/009903

| C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT |  |                       |
|--|--|-----------------------|
| Category *   | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
| A  | <p>JOHNSON L A ET AL: "SEX PRESELECTION:<br/>HIGH-SPEED FLOW CYTOMETRIC SORTING OF X<br/>AND Y SPERM FOR MAXIMUM EFFICIENCY"<br/>THERIOGENOLOGY, LOS ALTOS, CA, US,<br/>vol. 52, no. 8, 1999, pages 1323-1341,<br/>XP001023997<br/>ISSN: 0093-691X<br/>p. 1327, lines 1 - 4, p. 1328, lines 18 -<br/>38</p> <p style="text-align: center;">-----</p> | 1-38                  |
| A  | <p>WO 02/41906 A (WOODARD SCOTT ; BASKIN<br/>JAMES (US); DIDION BRADLEY (US); PHARMACIA<br/>CORP) 30 May 2002 (2002-05-30)<br/>p. 2, lines 4 - 17p. 3, line 28 - p. 4,<br/>line 5, p. 15, lines 11 - 21, Examples 2 -<br/>5</p> <p style="text-align: center;">-----</p>   | 1-38                  |

# INTERNATIONAL SEARCH REPORT

formation on patent family members

International Application No

PCT/US2004/009903

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s) | Publication<br>date         |
|---|---------------------|----------------------------|-----------------------------|
| WO 0151612                                | A                   | 19-07-2001                 | IT MI20000030 A1 16-07-2001 |
|   |                     | AU 3017601 A               | 24-07-2001                  |
|   |                     | WO 0151612 A1              | 19-07-2001                  |
|   |                     | EP 1255812 A1              | 13-11-2002                  |
| WO 0241906                                | A                   | 30-05-2002                 | AU 1980202 A 03-06-2002     |
|   |                     | CA 2454340 A1              | 30-05-2002                  |
|   |                     | WO 0241906 A2              | 30-05-2002                  |